(54) Title: HLA BINDING PEPTIDES AND THEIR USES

(57) Abstract

The present invention provides peptide compositions capable of specifically binding selected MHC alleles and inducing T cell activation in T cells restricted by the MHC allele. The peptides are useful to elicit an immune response against a desired antigen.

HLA BINDING PEPTIDES AND THEIR USES

The present application is a continuation in part of
USSN 08/027,746 which is a continuation in part of USSN
07/926,666, which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to compositions and
methods for preventing, treating or diagnosing a number of
pathological states such as viral diseases and cancers. In
particular, it provides novel peptides capable of binding
selected major histocompatibility complex (MHC) molecules and
inducing an immune response.

MHC molecules are classified as either Class I or
Class II molecules. Class II MHC molecules are expressed
primarily on cells involved in initiating and sustaining
immune responses, such as T lymphocytes, B lymphocytes,
macrophages, etc. Class II MHC molecules are recognized by
helper T lymphocytes and induce proliferation of helper T
lymphocytes and amplification of the immune response to the
particular immunogenic peptide that is displayed. Class I MHC
molecules are expressed on almost all nucleated cells and are
recognized by cytotoxic T lymphocytes (CTLs), which then
destroy the antigen-bearing cells. CTLs are particularly
important in tumor rejection and in fighting viral infections.
The CTL recognizes the antigen in the form of a peptide
fragment bound to the MHC class I molecules rather than the
intact foreign antigen itself. The antigen must normally be
endogenously synthesized by the cell, and a portion of the
protein antigen is degraded into small peptide fragments in
the cytoplasm. Some of these small peptides translocate into
a pre-Golgi compartment and interact with class I heavy chains
to facilitate proper folding and association with the subunit
B2 microglobulin. The peptide-MHC class I complex is then
routed to the cell surface for expression and potential
recognition by specific CTLs.

2

Investigations of the crystal structure of the human
MHC class I molecule, HLA-A2.1, indicate that a peptide
binding groove is created by the folding of the $\alpha 1$ and $\alpha 2$
domains of the class I heavy chain (Bjorkman et al., _Nature_
5   329:506 (1987). In these investigations, however, the
identity of peptides bound to the groove was not determined.
Buus et al., _Science_ 242:1065 (1988) first described
a method for acid elution of bound peptides from MHC.
Subsequently, Rammensee and his coworkers (Falk et al., _Nature_
10   351:290 (1991) have developed an approach to characterize
naturally processed peptides bound to class I molecules.
Other investigators have successfully achieved direct amino
acid sequencing of the more abundant peptides in various HPLC
fractions by conventional automated sequencing of peptides
15   eluted from class I molecules of the B type (Jardetzky, et
al., _Nature_ 353:326 (1991) and of the A2.1 type by mass
spectrometry (Hunt, et al., _Science_ 225:1261 (1992). A review
of the characterization of naturally processed peptides in MHC
Class I has been presented by Rötzschke and Falk (Rötzschke
20   and Falk, _Immunol. Today_ 12:447 (1991).

Sette et al., _Proc. Natl. Acad. Sci. USA_ 86:3296
(1989) showed that MHC allele specific motifs could be used to
predict MHC binding capacity. Schaeffer et al., _Proc. Natl._
Acad. Sci. USA 86:4649 (1989) showed that MHC binding was
25   related to immunogenicity. Several authors (De Bruijn et al.,
Eur. J. Immunol., 21:2963-2970 (1991); Pamer et al., 991
Nature 353:852-955 (1991)) have provided preliminary evidence
that class I binding motifs can be applied to the
identification of potential immunogenic peptides in animal
30   models. Class I motifs specific for a number of human alleles
of a given class I isotype have yet to be described. It is
desirable that the combined frequencies of these different
alleles should be high enough to cover a large fraction or
perhaps the majority of the human outbred population.

35   Despite the developments in the art, the prior art
has yet to provide a useful human peptide-based vaccine or
therapeutic agent based on this work. The present invention
provides these and other advantages.

3

SUMMARY OF THE INVENTION

The present invention provides compositions comprising immunogenic peptides having binding motifs for MHC Class I molecules. The immunogenic peptides are typically between about 8 and about 11 residues and comprise conserved residues involved in binding proteins encoded by the appropriate MHC allele. A number of allele specific motifs have been identified.

For instance, the motif for HLA-A3.2 comprises from the N-terminus to C-terminus a first conserved residue of L, M, I, V, S, A, T and F at position 2 and a second conserved residue of K, R or Y at the C-terminal end. Other first conserved residues are C, G or D and alternatively E. Other second conserved residues are H or F. The first and second conserved residues are preferably separated by 6 to 7 residues.

The motif for HLA-A1 comprises from the N-terminus to the C-terminus a first conserved residue of T, S or M, a second conserved residue of D or E, and a third conserved residue of Y. Other second conserved residues are A, S or T. The first and second conserved residues are adjacent and are preferably separated from the third conserved residue by 6 to 7 residues. A second motif consists of a first conserved residue of E or D and a second conserved residue of Y where the first and second conserved residues are separated by 5 to 6 residues.

The motif for HLA-A11 comprises from the N-terminus to the C-terminus a first conserved residue of T or V at position 2 and a C-terminal conserved residue of K. The first and second conserved residues are preferably separated by 6 or 7 residues.

The motif for HLA-A24.1 comprises from the N-terminus to the C-terminus a first conserved residue of Y, F or W at position 2 and a C terminal conserved residue of F, I, W, M or L. The first and second conserved residues are preferably separated by 6 to 7 residues.

Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable

4

antigens include prostate specific antigen (PSA), hepatitis B
core and surface antigens (HBVc, HBVs) hepatitis C antigens,
malignant melanoma antigen (MAGE-1) Epstein-Barr virus
antigens, human immunodeficiency type-1 virus (HIV1) and
papilloma virus antigens. The peptides are thus useful in
pharmaceutical compositions for both _in vivo_ and _ex vivo_
therapeutic and diagnostic applications.

Definitions

The term "peptide" is used interchangeably with
"oligopeptide" in the present specification to designate a
series of residues, typically L-amino acids, connected one to
the other typically by peptide bonds between the alpha-amino
and carbonyl groups of adjacent amino acids. The
oligopeptides of the invention are less than about 15 residues
in length and usually consist of between about 8 and about 11
residues, preferably 9 or 10 residues.

An "immunogenic peptide" is a peptide which
comprises an allele-specific motif such that the peptide will
bind the MHC allele and be capable of inducing a CTL response.
Thus, immunogenic peptides are capable of binding to an
appropriate class I MHC molecule and inducing a cytotoxic T
cell response against the antigen from which the immunogenic
peptide is derived.

A "conserved residue" is an amino acid which occurs
in a significantly higher frequency than would be expected by
random distribution at a particular position in a peptide
motif. Typically a conserved residue is one at which the
immunogenic peptide may provide a contact point with the MHC
molecule. One to three, preferably two, conserved residues
within a peptide of defined length defines a motif for an
immunogenic peptide. These residues are typically in close
contact with the peptide binding groove, with their side
chains buried in specific pockets of the groove itself.
Typically, an immunogenic peptide will comprise up to three
conserved residues, more usually two conserved residues.

As used herein, "negative binding residues" are
amino acids which if present at certain positions will result

5

in a peptide being a nonbinder or poor binder and in turn fail
to induce a CTL response despite the presence of the
appropriate conserved residues within the peptide.

5   The term "motif" refers to the pattern of residues
in a peptide of defined length, usually about 8 to about 11
amino acids, which is recognized by a particular MHC allele.
The peptide motifs are typically different for each human MHC
allele and differ in the pattern of the highly conserved
residues.

10   The binding motif for an allele can be defined with
increasing degrees of precision. In one case, all of the
conserved residues are present in the correct positions in a
peptide and there are no negative binding residues present.

        The phrases "isolated" or "biologically pure" refer
15   to material which is substantially or essentially free from
components which normally accompany it as found in its native
state. Thus, the peptides of this invention do not contain
materials normally associated with their in situ environment,
e.g., MHC I molecules on antigen presenting cells. Even where
20   a protein has been isolated to a homogenous or dominant band,
there are trace contaminants in the range of 5-10% of native
protein which co-purify with the desired protein. Isolated
peptides of this invention do not contain such endogenous co-
purified protein.

25   The term "residue" refers to an amino acid or amino
acid mimetic incorporated in an oligopeptide by an amide bond
or amide bond mimetic.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram of an HLA-A purification scheme.

5   Fig. 2 is an SDS-PAGE analysis of affinity purified. HLA-A3.2 from the cell line EHM using an affinity column prepared with the mAb GAP A3 coupled to protein A-Sepharose.

Lane 1 - Molecular weight standards.

Lane 2 - A3.2 acid eluate

10   Lane 3 - A3.2 a second acid eluate

Lane 4 - Base elution #1

Lane 5 - Base elution #2

Lane 6 - Concentrated base elution 1

Lane 7 - Concentrated base elution 2

15   Lane 8 - BSA - 10 μg

Lane 9 - BSA - 3 μg

Lane 10 - BSA - 1 μg

Fig. 3 shows reverse phase high performance liquid chromatography (RP-HPLC) separation of HLA-A3 acid eluted 20 peptides.

Fig. 4 shows binding of a radioactively labeled peptide of the invention to MHC molecules as measured by the % bound radioactivity.

Fig. 5 shows inhibition of binding of a peptide of 25 the invention to MHC molecules in the presence of three peptides [HBc 18-27 (924.07), a Prostate Specific Antigen peptide (939.01), and HIV nef 73-82 (940.03)].

Fig. 6 shows the dependency of the binding on MHC concentration in the presence or absence of β_2 microglobulin.

30   Fig. 7 shows dose dependent inhibition of binding with the addition of unlabeled peptide.

Fig. 8 Scatchard Analysis of binding to MHC A11 confirming an apparent K_D of 6nM.

Fig. 9 shows the binding of a radioactively labeled 35 peptide of the invention to MHC A1 as measured by % bound reactivity.

Fig. 10 shows dose dependent inhibition of binding with the addition of unlabeled peptide.

7

Fig. 11 Scatchard Analysis of binding to MHC A1 confirming an apparent K_D of 21nM.

Fig. 12 shows the binding of two peptides of this invention as a function of MHC A24 concentration as measured by % bound reactivity.

Fig. 13 shows the dose dependent inhibition of binding to MHC A24 with the addition of unlabeled peptides.

Figs. 14(a) and 14(b) show the Scatchard Analysis of binding to MHC A24 of the two peptides confirming a K_D of 30 and 60nM, respectively.

Fig. 15 shows the effect on MHC class 1 molecules of $\beta_2$microglobulin and a peptide of choice on acid-stripped PHA blasts.

Fig. 16 shows CTL induction using GC43 A2.1 responders and autologous acid-stripped PBMCs or PHA blasts loaded with the 777.03-924.07-927.32 peptide pool.

Fig. 17 shows CTL induction using X351 or X355 A2.1 responders and autologous acid stripped PBMCs or PHA blasts as stimulators after loading with the 1044.04-1044.05-1044.06 peptide pool.

Fig. 18 shows CTL induction using GC49 A2.1 responders and Autologous Acid stripped PHA blasts as stimulators after loading with 939.03 peptide.

Fig. 19 shows CTL induction using GC66 A1 responders and autologous acid stripped PBMCs as stimulators after loading of peptide 938.01.

Fig. 20 illustrates the lysis of peptide sensitized targets and endogenous targets following stimulation with SAC-I activated PBMCs loaded with a MAGE 3 peptide.

Fig. 21 shows a comparison of the acid strip loading with the cold temperature incubation.

Fig. 22 shows a CTL response to an immunogenic peptide for MAGE/A11.

Fig. 23 shows a CTL response to an immunogenic peptide for HIV/A3.

Fig. 24 shows a CTL response to an immunogenic peptide for HCV/A3.

8

Fig. 25 shows a CTL response to an immunogenic peptide for HBV/A1.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the determination of allele-specific peptide motifs for human Class I MHC (sometimes referred to as HLA) allele subtypes. These motifs are then used to define T cell epitopes from any desired antigen, particularly those associated with human viral diseases or cancers, for which the amino acid sequence of the potential antigen targets is known.

Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (e.g., MAGE-1), human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens.

Peptides comprising these epitopes are synthesized and then tested for their ability to bind to the appropriate MHC molecules in assays using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorimetry, peptide-dependent class I assembly assays, and inhibition of CTL recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary in vitro or in vivo CTL responses that can give rise to CTL populations capable of reacting with virally infected target cells or tumor cells as potential therapeutic agents.

The MHC class I antigens are encoded by the HLA-A, B, and C loci. HLA-A and B antigens are expressed at the cell surface at approximately equal densities, whereas the expression of HLA-C is significantly lower (perhaps as much as 10-fold lower). Each of these loci have a number of alleles.

9

The peptide binding motifs of the invention are relatively
specific for each allelic subtype.

   For peptide-based vaccines, the peptides of the
present invention preferably comprise a motif recognized by an
5   MHC I molecule having a wide distribution in the human
population. Since the MHC alleles occur at different
frequencies within different ethnic groups and races, the
choice of target MHC allele may depend upon the target
population. Table 1 shows the frequency of various alleles at
10   the HLA-A locus products among different races. For instance,
the majority of the Caucasoid population can be covered by
peptides which bind to four HLA-A allele subtypes,
specifically HLA-A2.1, A1, A3.2, and A24.1. Similarly, the
majority of the Asian population is encompassed with the
15   addition of peptides binding to a fifth allele HLA-A11.2.

TABLE 1

A Allele/Subtype	N(69)*	A(54)	C(502)
A1	10.1(7)	1.8(1)	27.4(138)
A2.1	11.5(8)	37.0(20)	39.8(199)
A2.2	10.1(7)	0	3.3(17)
A2.3	1.4(1)	5.5(3)	0.8(4)
A2.4	-	-	-
A2.5	-	-	-
A3.1	1.4(1)	0	0.2(0)
A3.2	5.7(4)	5.5(3)	21.5(108)
A11.1	0	5.5(3)	0
A11.2	5.7(4)	31.4(17)	8.7(44)
A11.3	0	3.7(2)	0
A23	4.3(3)	-	3.9(20)
A24	2.9(2)	27.7(15)	15.3(77)
A24.2	-	-	-
A24.3	-	-	-
A25	1.4(1)	-	6.9(35)
A26.1	4.3(3)	9.2(5)	5.9(30)
A26.2	7.2(5)	-	1.0(5)
A26V	-	3.7(2)	-
A28.1	10.1(7)	-	1.6(8)
A28.2	1.4(1)	-	7.5(38)
A29.1	1.4(1)	-	1.4(7)
A29.2	10.1(7)	1.8(1)	5.3(27)
A30.1	8.6(6)	-	4.9(25)
A30.2	1.4(1)	-	0.2(1)
A30.3	7.2(5)	-	3.9(20)
A31	4.3(3)	7.4(4)	6.9(35)
A32	2.8(2)	-	7.1(36)
Aw33.1	8.6(6)	-	2.5(13)
Aw33.2	2.8(2)	16.6(9)	1.2(6)
Aw34.1	1.4(1)	-	-
Aw34.2	14.5(10)	-	0.8(4)
Aw36	5.9(4)	-	-

Table compiled from B. DuPont, Immunobiology of HLA, Vol.
I, Histocompatibility Testing 1987, Springer-Verlag, New York
1989.

* N - negroid; A = Asian; C = caucasoid. Numbers in
   parenthesis represent the number of individuals included in
the analysis.

          The nomenclature used to describe peptide compounds
follows the conventional practice wherein the amino group is
presented to the left (the N-terminus) and the carboxyl group
to the right (the C-terminus) of each amino acid residue. In
the formulae representing selected specific embodiments of the
present invention, the amino- and carboxyl-terminal groups,
although not specifically shown, are in the form they would

assume at physiologic pH values, unless otherwise specified.
In the amino acid structure formulae, each residue is
generally represented by standard three letter or single
letter designations. The L-form of an amino acid residue is
represented by a capital single letter or a capital first
letter of a three-letter symbol, and the D-form for those
amino acids is represented by a lower case single letter or a
lower case three letter symbol. Glycine has no asymmetric
carbon atom and is simply referred to as "Gly" or G.

        The procedures used to identify peptides of the
present invention generally follow the methods disclosed in
Falk et al., Nature 351:290 (1991), which is incorporated
herein by reference. Briefly, the methods involve large-scale
isolation of MHC class I molecules, typically by
immunoprecipitation or affinity chromatography, from the
appropriate cell or cell line. Examples of other methods for
isolation of the desired MHC molecule equally well known to
the artisan include ion exchange chromatography, lectin
chromatography, size exclusion, high performance ligand
chromatography, and a combination of all of the above
techniques.

        A large number of cells with defined MHC molecules,
particularly MHC Class I molecules, are known and readily
available. For example, human EBV-transformed B cell lines
have been shown to be excellent sources for the preparative
isolation of class I and class II MHC molecules.
Well-characterized cell lines are available from private and
commercial sources, such as American Type Culture Collection
("Catalogue of Cell Lines and Hybridomas," 6th edition (1988)
Rockville, Maryland, U.S.A.); National Institute of General
Medical Sciences 1990/1991 Catalog of Cell Lines (NIGMS) Human
Genetic Mutant Cell Repository, Camden, NJ; and ASHI
Repository, Bingham and Women's Hospital, 75 Francis Street,
Boston, MA 02115. Table 2 lists some B cell lines suitable
for use as sources for HLA-A alleles. All of these cell lines
can be grown in large batches and are therefore useful for
large scale production of MHC molecules. One of skill will
recognize that these are merely exemplary cell lines and that

12

many other cell sources can be employed. Similar EBV B cell
lines homozygous for HLA-B and HLA-C could serve as sources
for HLA-B and HLA-C alleles, respectively.

13

TABLE 2

HUMAN CELL LINES (HLA-A SOURCES)

HLA-A allele	B cell line
A1	MAT COX (9022) STEINLIN (9087)
A2.1	JY
A3.2	EHM (9080) HO301 (9055)GM3107
A24.1	KT3(9107),TISI (9042)
A11	BVR (GM6828A) WT100 (GM8602),WT52 (GM8603)

In the typical case, immunoprecipitation is used to isolate the desired allele. A number of protocols can be used, depending upon the specificity of the antibodies used. For example, allele-specific mAb reagents can be used for the affinity purification of the HLA-A, HLA-B, and HLA-C molecules. Several mAb reagents for the isolation of HLA-A molecules are available (Table 3). Thus, for each of the targeted HLA-A alleles, reagents are available that may be used for the direct isolation of the HLA-A molecules. Affinity columns prepared with these mAbs using standard techniques are successfully used to purify the respective HLA-A allele products.

In addition to allele-specific mAbs, broadly reactive anti-HLA-A, B, C mAbs, such as W6/32 and B9.12.1, and

14

one anti-HLA-B, C mAb, B1.23.2, could be used in alternative affinity purification protocols as described in the example section below.

TABLE 3

ANTIBODY REAGENTS

anti-HLA	Name
HLA-A1	12/18
HLA-A3	GAPA3 (ATCC, HB122)
HLA-11,24.1	A11.1M (ATCC, HB164)
HLA-A,B,C	W6/32 (ATCC, HB95)
monomorphic	B9.12.1 (INSERM-CNRS)
HLA-B,C	B.1.23.2 (INSERM-CNRS)
monomorphic	

The peptides bound to the peptide binding groove of the isolated MHC molecules are eluted typically using acid treatment. Peptides can also be dissociated from class I molecules by a variety of standard denaturing means, such as heat, pH, detergents, salts, chaotropic agents, or a combination thereof.

Peptide fractions are further separated from the MHC molecules by reversed-phase high performance liquid chromatography (HPLC) and sequenced. Peptides can be separated by a variety of other standard means well known to the artisan, including filtration, ultrafiltration, electrophoresis, size chromatography, precipitation with specific antibodies, ion exchange chromatography, isoelectrofocusing, and the like.

Sequencing of the isolated peptides can be performed according to standard techniques such as Edman degradation (Hunkapiller, M.W., et al., Methods Enzymol. 91, 399 [1983]). Other methods suitable for sequencing include mass spectrometry sequencing of individual peptides as previously described (Hunt, et al., Science 225:1261 (1992), which is incorporated herein by reference). Amino acid sequencing of bulk heterogenous peptides (e.g., pooled HPLC fractions) from different class I molecules typically reveals a characteristic sequence motif for each class I allele.

Definition of motifs specific for different class I alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino acid sequence is known. Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs. The epitopic sequences are then synthesized. The capacity to bind MHC Class molecules is measured in a variety of different ways. One means is a Class I molecular binding assay as described in Example 10, below. Other alternatives described in the literature include inhibition of antigen presentation (Sette, et al., J. Immunol. 141:3893 (1991), in vitro assembly assays (Townsend, et al., Cell 62:285 (1990), and FACS based assays using mutated ells, such as RMA.S (Melief, et al., Eur. J. Immunol. 21:2963 [1991]).

Next, peptides that test positive in the MHC class I binding assay are assayed for the ability of the peptides to induce specific CTL responses in vitro. For instance, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., J. Exp. Med. 166:182 (1987); Boog, Eur. J. Immunol. 18:219 [1988]).

Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides, such as the mouse cell lines RMA-S (Kärre, et al.. Nature, 319:675 (1986); Ljunggren, et

16

al., _Eur. J. Immunol._ 21:2963-2970 (1991)), and the human
somatic T cell hybridoma, T-2 (Cerundolo, et al., _Nature_
345:449-452 (1990)) and which have been transfected with the
appropriate human class I genes are conveniently used, when
peptide is added to them, to test for the capacity of the
peptide to induce _in vitro_ primary CTL responses. Other
eukaryotic cell lines which could be used include various
insect cell lines such as mosquito larvae (ATCC cell lines CCL
125, 126, 1660, 1591, 6585, 6586), silkworm (ATTC CRL 8851),
armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila
cell lines such as a Schneider cell line (see Schneider _J._
Embryol. Exp. Morphol. 27:353-365 [1927]). That have been
transfected with the appropriate human class I MHC allele
encoding genes and the human B_2 microglobulin genes.

          Peripheral blood lymphocytes are conveniently
isolated following simple venipuncture or leukapheresis of
normal donors or patients and used as the responder cell
sources of CTL precursors. In one embodiment, the appropriate
antigen-presenting cells are incubated with 10-100 μM of
peptide in serum-free media for 4 hours under appropriate
culture conditions. The peptide-loaded antigen-presenting
cells are then incubated with the responder cell populations
in vitro for 7 to 10 days under optimized culture conditions.
Positive CTL activation can be determined by assaying the
cultures for the presence of CTLs that kill radiolabeled
target cells, both specific peptide-pulsed targets as well as
target cells expressing endogenously processed form of the
relevant virus or tumor antigen from which the peptide
sequence was derived.

          Specificity and MHC restriction of the CTL is
determined by testing against different peptide target cells
expressing appropriate or inappropriate human MHC class I.
The peptides that test positive in the MHC binding assays and
give rise to specific CTL responses are referred to herein as
immunogenic peptides.

          The immunogenic peptides can be prepared
synthetically, or by recombinant DNA technology or isolated
from natural sources such as whole viruses or tumors.

17

Although the peptide will preferably be substantially free of
other naturally occurring host cell proteins and fragments
thereof, in some embodiments the peptides can be synthetically
conjugated to native fragments or particles. The polypeptides
5   or peptides can be a variety of lengths, either in their
neutral (uncharged) forms or in forms which are salts, and
either free of modifications such as glycosylation, side chain
oxidation, or phosphorylation or containing these
modifications, subject to the condition that the modification
10   not destroy the biological activity of the polypeptides as
herein described.

        Desirably, the peptide will be as small as possible
while still maintaining substantially all of the biological
activity of the large peptide. When possible, it may be
15   desirable to optimize peptides of the invention to a length of
9 or 10 amino acid residues, commensurate in size with
endogenously processed viral peptides or tumor cell peptides
that are bound to MHC class I molecules on the cell surface.

        Peptides having the desired activity may be modified
20   as necessary to provide certain desired attributes, e.g.,
improved pharmacological characteristics, while increasing or
at least retaining substantially all of the biological
activity of the unmodified peptide to bind the desired MHC
molecule and activate the appropriate T cell. For instance,
25   the peptides may be subject to various changes, such as
substitutions, either conservative or non-conservative, where
such changes might provide for certain advantages in their
use, such as improved MHC binding. By conservative
substitutions is meant replacing an amino acid residue with
30   another which is biologically and/or chemically similar, e.g.,
one hydrophobic residue for another, or one polar residue for
another. The substitutions include combinations such as Gly,
Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys,
Arg; and Phe, Tyr. The effect of single amino acid
35   substitutions may also be probed using D-amino acids. Such
modifications may be made using well known peptide synthesis
procedures, as described in e.g., Merrifield, Science 232:341-
347 (1986), Barany and Merrifield, The Peptides, Gross and

Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and
Stewart and Young, Solid Phase peptide Synthesis, (Rockford,
Ill., Pierce), 2d Ed. (1984), incorporated by reference
herein.

5   The peptides can also be modified by extending or
decreasing the compound's amino acid sequence, e.g., by the
addition or deletion of amino acids. The peptides or analogs
of the invention can also be modified by altering the order or
composition of certain residues, it being readily appreciated
10   that certain amino acid residues essential for biological
activity, e.g., those at critical contact sites or conserved
residues, may generally not be altered without an adverse
effect on biological activity. The non-critical amino acids
need not be limited to those naturally occurring in proteins,
15   such as L-α-amino acids, or their D-isomers, but may include
non-natural amino acids as well, such as β-γ-δ-amino acids, as
well as many derivatives of L-α-amino acids.
          Typically, a series of peptides with single amino
acid substitutions are employed to determine the effect of
20   electrostatic charge, hydrophobicity, etc. on binding. For
instance, a series of positively charged (e.g., Lys or Arg) or
negatively charged (e.g., Glu) amino acid substitutions are
made along the length of the peptide revealing different
patterns of sensitivity towards various MHC molecules and T
25   cell receptors. In addition, multiple substitutions using
small, relatively neutral moieties such as Ala, Gly, Pro, or
similar residues may be employed. The substitutions may be
homo-oligomers or hetero-oligomers. The number and types of
residues which are substituted or added depend on the spacing
30   necessary between essential contact points and certain
functional attributes which are sought (e.g., hydrophobicity
versus hydrophilicity). Increased binding affinity for an MHC
molecule or T cell receptor may also be achieved by such
substitutions, compared to the affinity of the parent peptide.
35   In any event, such substitutions should employ amino acid
residues or other molecular fragments chosen to avoid, for
example, steric and charge interference which might disrupt
binding.

Amino acid substitutions are typically of single
residues. Substitutions, deletions, insertions or any
combination thereof may be combined to arrive at a final
peptide. Substitutional variants are those in which at least
5   one residue of a peptide has been removed and a different
residue inserted in its place. Such substitutions generally
are made in accordance with the following Table 4 when it is
desired to finely modulate the characteristics of the peptide.

TABLE 4

Original Residue	Exemplary Substitution
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

Substantial changes in function (e.g., affinity for MHC molecules or T cell receptors) are made by selecting substitutions that are less conservative than those in Table 4, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in peptide properties will be those in which (a) hydrophilic residue, e.g. seryl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (c) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

The peptides may also comprise isosteres of two or more residues in the immunogenic peptide. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, Chemistry and Biochemistry of Amino Acids, peptides and Proteins, Vol. VII (Weinstein ed., 1983).

Modifications of peptides with various amino acid mimetics or unnatural amino acids are particularly useful in increasing the stability of the peptide in vivo. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al.,

Eur. J. Drug Metab Pharmacokin. 11:291-302 (1986). Half life
of the peptides of the present invention is conveniently
determined using a 25% human serum (v/v) assay. The protocol
is generally as follows. Pooled human serum (Type AB,

5   non-heat inactivated) is delipidated by centrifugation before
use. The serum is then diluted to 25% with RPMI tissue
culture media and used to test peptide stability. At
predetermined time intervals a small amount of reaction
solution is removed and added to either 6% aqueous

10   trichloracetic acid or ethanol. The cloudy reaction sample is
cooled (4°C) for 15 minutes and then spun to pellet the
precipitated serum proteins. The presence of the peptides is
then determined by reversed-phase HPLC using
stability-specific chromatography conditions.

15   The peptides of the present invention or analogs
thereof which have CTL stimulating activity may be modified to
provide desired attributes other than improved serum half
life. For instance, the ability of the peptides to induce CTL
activity can be enhanced by linkage to a sequence which

20   contains at least one epitope that is capable of inducing a T
helper cell response. Particularly preferred immunogenic
peptides/T helper conjugates are linked by a spacer molecule.
The spacer is typically comprised of relatively small, neutral
molecules, such as amino acids or amino acid mimetics, which

25   are substantially uncharged under physiological conditions.
The spacers are typically selected from, e.g., Ala, Gly, or
other neutral spacers of nonpolar amino acids or neutral polar
amino acids. It will be understood that the optionally
present spacer need not be comprised of the same residues and

30   thus may be a hetero- or homo-oligomer. When present, the
spacer will usually be at least one or two residues, more
usually three to six residues. Alternatively, the CTL peptide
may be linked to the T helper peptide without a spacer.

          The immunogenic peptide may be linked to the T helper
35   peptide either directly or via a spacer either at the amino or
carboxy terminus of the CTL peptide. The amino terminus of
either the immunogenic peptide or the T helper peptide may be
acylated.

In some embodiments it may be desirable to include in
the pharmaceutical compositions of the invention at least one
component which assists in priming CTL. Lipids have been
identified as agents capable of assisting the priming CTL _in_
vivo against viral antigens. For example, palmitic acid
residues can be attached to the alpha and epsilon amino groups
of a Lys residue and then linked, e.g., via one or more
linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the
like, to an immunogenic peptide. The lipidated peptide can
then be injected directly in a micellar form, incorporated
into a liposome or emulsified in an adjuvant, e.g., incomplete
Freund's adjuvant. In a preferred embodiment a particularly
effective immunogen comprises palmitic acid attached to alpha
and epsilon amino groups of Lys, which is attached via
linkage, e.g., Ser-Ser, to the amino terminus of the
immunogenic peptide.

As another example of lipid priming of CTL responses,
E. coli lipoproteins, such as
tripalmitoyl-S-glycerylcysteinlyseryl-serine (P_3CSS) can be
used to prime virus specific CTL when covalently attached to
an appropriate peptide. See, Deres et al., _Nature_ 342:561-564
(1989), incorporated herein by reference. Peptides of the
invention can be coupled to P_3CSS, for example, and the
lipopeptide administered to an individual to specifically
prime a CTL response to the target antigen. Further, as the
induction of neutralizing antibodies can also be primed with
P_3CSS conjugated to a peptide which displays an appropriate
epitope, the two compositions can be combined to more
effectively elicit both humoral and cell-mediated responses to
infection.

In addition, additional amino acids can be added to
the termini of a peptide to provide for ease of linking
peptides one to another, for coupling to a carrier support, or
larger peptide, for modifying the physical or chemical
properties of the peptide or oligopeptide, or the like. Amino
acids such as tyrosine, cysteine, lysine, glutamic or aspartic
acid, or the like, can be introduced at the C- or N-terminus
of the peptide or oligopeptide. Modification at the C

24

terminus in some cases may alter binding characteristics of
the peptide. In addition, the peptide or oligopeptide
sequences can differ from the natural sequence by being
modified by terminal-NH_2 acylation, e.g., by alkanoyl (C_1-C_{20})
or thioglycolyl acetylation, terminal-carboxyl amidation,
e.g., ammonia, methylamine, etc. In some instances these
modifications may provide sites for linking to a support or
other molecule.

        The peptides of the invention can be prepared in a
wide variety of ways. Because of their relatively short size,
the peptides can be synthesized in solution or on a solid
support in accordance with conventional techniques. Various
automatic synthesizers are commercially available and can be
used in accordance with known protocols. See, for example,
Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed.,
Pierce Chemical Co. (1984), supra.

        Alternatively, recombinant DNA technology may be
employed wherein a nucleotide sequence which encodes an
immunogenic peptide of interest is inserted into an expression
vector, transformed or transfected into an appropriate host
cell and cultivated under conditions suitable for expression.
These procedures are generally known in the art, as described
generally in Sambrook et al., Molecular Cloning. A Laboratory
Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York
(1982), which is incorporated herein by reference. Thus,
fusion proteins which comprise one or more peptide sequences
of the invention can be used to present the appropriate T cell
epitope.

        As the coding sequence for peptides of the length
contemplated herein can be synthesized by chemical techniques,
for example, the phosphotriester method of Matteucci et al.,
J. Am. Chem. Soc. 103:3185 (1981), modification can be made
simply by substituting the appropriate base(s) for those
encoding the native peptide sequence. The coding sequence can
then be provided with appropriate linkers and ligated into
expression vectors commonly available in the art, and the
vectors used to transform suitable hosts to produce the
desired fusion protein. A number of such vectors and suitable

host systems are now available. For expression of the fusion
proteins, the coding sequence will be provided with operably
linked start and stop codons, promoter and terminator regions
and usually a replication system to provide an expression
5   vector for expression in the desired cellular host. For
example, promoter sequences compatible with bacterial hosts
are provided in plasmids containing convenient restriction
sites for insertion of the desired coding sequence. The
resulting expression vectors are transformed into suitable
10   bacterial hosts. Of course, yeast or mammalian cell hosts may
also be used, employing suitable vectors and control
sequences.

         The peptides of the present invention and
pharmaceutical and vaccine compositions thereof are useful for
15   administration to mammals, particularly humans, to treat
and/or prevent viral infection and cancer. Examples of
diseases which can be treated using the immunogenic peptides
of the invention include prostate cancer, hepatitis B,
hepatitis C, AIDS, renal carcinoma, cervical carcinoma,
20   lymphoma, CMV and condlyloma acuminatum.

         For pharmaceutical compositions, the immunogenic
peptides of the invention are administered to an individual
already suffering from cancer or infected with the virus of
interest. Those in the incubation phase or the acute phase of
25   infection can be treated with the immunogenic peptides
separately or in conjunction with other treatments, as
appropriate.   In therapeutic applications, compositions are
administered to a patient in an amount sufficient to elicit an
effective CTL response to the virus or tumor antigen and to
30   cure or at least partially arrest symptoms and/or
complications. An amount adequate to accomplish this is
defined as "therapeutically effective dose." Amounts
effective for this use will depend on, e.g., the peptide
composition, the manner of administration, the stage and
35   severity of the disease being treated, the weight and general
state of health of the patient, and the judgment of the
prescribing physician, but generally range for the initial
immunization (that is for therapeutic or prophylactic

administration) from about 1.0 µg to about 5000 µg of peptide
for a 70 kg patient, followed by boosting dosages of from
about 1.0 µg to about 1000 µg of peptide pursuant to a
boosting regimen over weeks to months depending upon the
5   patient's response and condition by measuring specific CTL
activity in the patient's blood. It must be kept in mind that
the peptides and compositions of the present invention may
generally be employed in serious disease states, that is,
life-threatening or potentially life threatening situations.
10   In such cases, in view of the minimization of extraneous
substances and the relative nontoxic nature of the peptides,
it is possible and may be felt desirable by the treating
physician to administer substantial excesses of these peptide
compositions.

15   For therapeutic use, administration should begin at
the first sign of viral infection or the detection or surgical
removal of tumors or shortly after diagnosis in the case of
acute infection. This is followed by boosting doses until at
least symptoms are substantially abated and for a period
20   thereafter. In chronic infection, loading doses followed by
boosting doses may be required.

                Treatment of an infected individual with the
compositions of the invention may hasten resolution of the
infection in acutely infected individuals. For those
25   individuals susceptible (or predisposed) to developing chronic
infection the compositions are particularly useful in methods
for preventing the evolution from acute to chronic infection.
Where the susceptible individuals are identified prior to or
during infection, for instance, as described herein, the
30   composition can be targeted to them, minimizing need for
administration to a larger population.

                The peptide compositions can also be used for the
treatment of chronic infection and to stimulate the immune
system to eliminate virus-infected cells in carriers. It is
35   important to provide an amount of immuno-potentiating peptide
in a formulation and mode of administration sufficient to
effectively stimulate a cytotoxic T cell response. Thus, for
treatment of chronic infection, a representative dose is in

27

the range of about 1.0 μg to about 5000 μg, preferably about 5 μg to 1000 μg for a 70 kg patient per dose. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

28

        The peptides of the invention may also be
administered via liposomes, which serve to target the peptides
to a particular tissue, such as lymphoid tissue, or targeted
selectively to infected cells, as well as increase the half-
5   life of the peptide composition. Liposomes include emulsions,
foams, micelles, insoluble monolayers, liquid crystals,
phospholipid dispersions, lamellar layers and the like. In
these preparations the peptide to be delivered is incorporated
as part of a liposome, alone or in conjunction with a molecule
10   which binds to, e.g., a receptor prevalent among lymphoid
cells, such as monoclonal antibodies which bind to the CD45
antigen, or with other therapeutic or immunogenic
compositions. Thus, liposomes filled with a desired peptide
of the invention can be directed to the site of lymphoid
15   cells, where the liposomes then deliver the selected
therapeutic/immunogenic peptide compositions. Liposomes for
use in the invention are formed from standard vesicle-forming
lipids, which generally include neutral and negatively charged
phospholipids and a sterol, such as cholesterol. The
20   selection of lipids is generally guided by consideration of,
e.g., liposome size, acid lability and stability of the
liposomes in the blood stream. A variety of methods are
available for preparing liposomes, as described in, e.g.,
Szoka et al., _Ann. Rev. Biophys. Bioeng_. 9:467 (1980), U.S.
25   Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369,
incorporated herein by reference.
        For targeting to the immune cells, a ligand to be
incorporated into the liposome can include, e.g., antibodies
or fragments thereof specific for cell surface determinants of
30   the desired immune system cells. A liposome suspension
containing a peptide may be administered intravenously,
locally, topically, etc. in a dose which varies according to,
inter alia, the manner of administration, the peptide being
delivered, and the stage of the disease being treated.
35   For solid compositions, conventional nontoxic solid
carriers may be used which include, for example,
pharmaceutical grades of mannitol, lactose, starch, magnesium
stearate, sodium saccharin, talcum, cellulose, glucose,

sucrose, magnesium carbonate, and the like. For oral
administration, a pharmaceutically acceptable nontoxic
composition is formed by incorporating any of the normally
employed excipients, such as those carriers previously listed,
and generally 10-95% of active ingredient, that is, one or
more peptides of the invention, and more preferably at a
concentration of 25%-75%.

        For aerosol administration, the immunogenic peptides
are preferably supplied in finely divided form along with a
surfactant and propellant. Typical percentages of peptides
are 0.01%-20% by weight, preferably 1%-10%. The surfactant
must, of course, be nontoxic, and preferably soluble in the
propellant. Representative of such agents are the esters or
partial esters of fatty acids containing from 6 to 22 carbon
atoms, such as caproic, octanoic, lauric, palmitic, stearic,
linoleic, linolenic, olesteric and oleic acids with an
aliphatic polyhydric alcohol or its cyclic anhydride. Mixed
esters, such as mixed or natural glycerides may be employed.
The surfactant may constitute 0.1%-20% by weight of the
composition, preferably 0.25-5%. The balance of the
composition is ordinarily propellant. A carrier can also be
included, as desired, as with, e.g., lecithin for intranasal
delivery.

        In another aspect the present invention is directed
to vaccines which contain as an active ingredient an
immunogenically effective amount of an immunogenic peptide as
described herein. The peptide(s) may be introduced into a
host, including humans, linked to its own carrier or as a
homopolymer or heteropolymer of active peptide units. Such a
polymer has the advantage of increased immunological reaction
and, where different peptides are used to make up the polymer,
the additional ability to induce antibodies and/or CTLs that
react with different antigenic determinants of the virus or
tumor cells. Useful carriers are well known in the art, and
include, e.g., thyroglobulin, albumins such as bovine serum
albumin, tetanus toxoid, polyamino acids such as
poly(lysine:glutamic acid), hepatitis B virus core protein,
hepatitis B virus recombinant vaccine and the like. The

vaccines can also contain a physiologically tolerable
(acceptable) diluent such as water, phosphate buffered saline,
or saline, and further typically include an adjuvant.
Adjuvants such as incomplete Freund's adjuvant, aluminum
5   phosphate, aluminum hydroxide, or alum are materials well
known in the art. And, as mentioned above, CTL responses can
be primed by conjugating peptides of the invention to lipids,
such as P_3CSS. Upon immunization with a peptide composition
as described herein, via injection, aerosol, oral, transdermal
10   or other route, the immune system of the host responds to the
vaccine by producing large amounts of CTLs specific for the
desired antigen, and the host becomes at least partially
immune to later infection, or resistant to developing chronic
infection.

15   Vaccine compositions containing the peptides of the
invention are administered to a patient susceptible to or
otherwise at risk of viral infection or cancer to elicit an
immune response against the antigen and thus enhance the
patient's own immune response capabilities. Such an amount is
20   defined to be an "immunogenically effective dose." In this
use, the precise amounts again depend on the patient's state
of health and weight, the mode of administration, the nature
of the formulation, etc., but generally range from about 1.0
μg to about 5000 μg per 70 kilogram patient, more commonly
25   from about 10 μg to about 500 mg per 70 kg of body weight.

            In some instances it may be desirable to combine the
peptide vaccines of the invention with vaccines which induce
neutralizing antibody responses to the virus of interest,
particularly to viral envelope antigens.

30   For therapeutic or immunization purposes, the
peptides of the invention can also be expressed by attenuated
viral hosts, such as vaccinia or fowlpox. This approach
involves the use of vaccinia virus as a vector to express
nucleotide sequences that encode the peptides of the
35   invention. Upon introduction into an acutely or chronically
infected host or into a non-infected host, the recombinant
vaccinia virus expresses the immunogenic peptide, and thereby
elicits a host CTL response. Vaccinia vectors and methods

31

useful in immunization protocols are described in, e.g.,U.S.
Patent No. 4,722,848, incorporated herein by reference.
Another vector is BCG (Bacille Calmette Guerin). BCG vectors
are described in Stover et al. (Nature 351:456-460 (1991))
which is incorporated herein by reference. A wide variety of
other vectors useful for therapeutic administration or
immunization of the peptides of the invention, e.g.,
Salmonella typhi vectors and the like, will be apparent to
those skilled in the art from the description herein.

Antigenic peptides may be used to elicit CTL ex vivo,
as well. The resulting CTL, can be used to treat chronic
infections (viral or bacterial) or tumors in patients that do
not respond to other conventional forms of therapy, or will
not respond to a peptide vaccine approach of therapy. Ex vivo
CTL responses to a particular pathogen (infectious agent or
tumor antigen) are induced by incubating in tissue culture the
patient's CTL precursor cells (CTLp) together with a source of
antigen-presenting cells (APC) and the appropriate immunogenic
peptide. After an appropriate incubation time (typically 1-4
weeks), in which the CTLp are activated and mature and expand
into effector CTL, the cells are infused back into the
patient, where they will destroy their specific target cell
(an infected cell or a tumor cell). In order to optimize the
in vitro conditions for the generation of specific cytotoxic T
cells, the culture of stimulator cells is maintained in an
appropriate serum-free medium.

Prior to incubation of the stimulator cells with the
cells to be activated, e.g., precursor CD8+ cells, an amount
of antigenic peptide is added to the stimulator cell culture,
of sufficient quantity to become loaded onto the human Class I
molecules to be expressed on the surface of the stimulator
cells. In the present invention, a sufficient amount of
peptide is an amount that will allow about 200, and preferably
200 or more, human Class I MHC molecules loaded with peptide
to be expressed on the surface of each stimulator cell.
Preferably, the stimulator cells are incubated with >20μg/ml
peptide.

Resting or precursor CD8+ cells are then incubated in
culture with the appropriate stimulator cells for a time
period sufficient to activate the CD8+ cells. Preferably, the
CD8+ cells are activated in an antigen-specific manner. The
ratio of resting or precursor CD8+ (effector) cells to
stimulator cells may vary from individual to individual and
may further depend upon variables such as the amenability of
an individual's lymphocytes to culturing conditions and the
nature and severity of the disease condition or other
condition for which the within-described treatment modality is
used. Preferably, however, the lymphocyte:stimulator cell
ratio is in the range of about 30:1 to 300:1. The
effector/stimulator culture may be maintained for as long a
time as is necessary to stimulate a therapeutically useable or
effective number of CD8+ cells.

The induction of CTL *in vitro* requires the specific
recognition of peptides that are bound to allele specific MHC
class I molecules on APC. The number of specific MHC/peptide
complexes per APC is crucial for the stimulation of CTL,
particularly in primary immune responses. While small amounts
of peptide/MHC complexes per cell are sufficient to render a
cell susceptible to lysis by CTL, or to stimulate a secondary
CTL response, the successful activation of a CTL precursor
(pCTL) during primary response requires a significantly higher
number of MHC/peptide complexes. Peptide loading of empty
major histocompatability complex molecules on cells allows the
induction of primary cytotoxic T lymphocyte responses. Peptide
loading of empty major histocompatability complex molecules on
cells enables the induction of primary cytotoxic T lymphocyte
responses.

Since mutant cell lines do not exist for every human
MHC allele, it is advantageous to use a technique to remove
endogenous MHC-associated peptides from the surface of APC,
followed by loading the resulting empty MHC molecules with the
immunogenic peptides of interest. The use of non-transformed
(non-tumorigenic), non-infected cells, and preferably,
autologous cells of patients as APC is desirable for the
design of CTL induction protocols directed towards development

of *ex vivo* CTL therapies. This application discloses methods
for stripping the endogenous MHC-associated peptides from the
surface of APC followed by the loading of desired peptides.

A stable MHC class I molecule is a trimeric complex
formed of the following elements: 1) a peptide usually of 8 -
10 residues, 2) a transmembrane heavy polymorphic protein
chain which bears the peptide-binding site in its α1 and α2
domains, and 3) a non-covalently associated non-polymorphic
light chain, $\beta_2$microglobulin. Removing the bound peptides
and/or dissociating the $\beta_2$microglobulin from the complex
renders the MHC class I molecules nonfunctional and unstable,
resulting in rapid degradation. All MHC class I molecules
isolated from PBMCs have endogenous peptides bound to them.
Therefore, the first step is to remove all endogenous peptides
bound to MHC class I molecules on the APC without causing
their degradation before exogenous peptides can be added to
them.

Two possible ways to free up MHC class I molecules of
bound peptides include lowering the culture temperature from
37°C to 26°C overnight to destablize $\beta_2$microglobulin and
stripping the endogenous peptides from the cell using a mild
acid treatement. The methods release previously bound
peptides into the extracellular environment allowing new
exogenous peptides to bind to the empty class I molecules.
The cold-temperature incubation method enables exogenous
peptides to bind efficiently to the MHC complex, but requires
an overnight incubation at 26°C which may slow the cell's
metabolic rate. It is also likely that cells not actively
synthesizing MHC molecules (e.g., resting PBMC) would not
produce high amounts of empty surface MHC molecules by the
cold temperature procedure.

Harsh acid stripping involves extraction of the
peptides with trifluoroacetic acid, pH 2, or acid denaturation
of the immunoaffinity purified class I-peptide complexes.
These methods are not feasible for CTL induction, since it is
important to remove the endogenous peptides while preserving
APC viability and an optimal metabolic state which is critical
for antigen presentation. Mild acid solutions of pH 3 such as

34

glycine or citrate-phosphate buffers have been used to
identify endogenous peptides and to identify tumor associated
T cell epitopes. The treatment is especially effective, in
that only the MHC class I molecules are destabilized (and

5   associated peptides released), while other surface antigens
remain intact, including MHC class II molecules. Most
importantly, treatment of cells with the mild acid solutions
do not affect the cell's viability or metabolic state. The
mild acid treatment is rapid since the stripping of the

10   endogenous peptides occurs in two minutes at 4°C and the APC
is ready to perform its function after the appropriate
peptides are loaded. The technique is utilized herein to make
peptide-specific APCs for the generation of primary
antigen-specific CTL. The resulting APC are efficient in

15   inducing peptide-specific CD8+ CTL.

            Activated CD8+ cells may be effectively separated
from the stimulator cells using one of a variety of known
methods. For example, monoclonal antibodies specific for the
stimulator cells, for the peptides loaded onto the stimulator

20   cells, or for the CD8+ cells (or a segment thereof) may be
utilized to bind their appropriate complementary ligand.
Antibody-tagged molecules may then be extracted from the
stimulator-effector cell admixture via appropriate means,
e.g., via well-known immunoprecipitation or immunoassay

25   methods.

            Effective, cytotoxic amounts of the activated CD8+
cells can vary between in vitro and in vivo uses, as well as
with the amount and type of cells that are the ultimate target
of these killer cells. The amount will also vary depending on

30   the condition of the patient and should be determined via
consideration of all appropriate factors by the practitioner.
Preferably, however, about 1×10^6 to about 1×10^{12}, more
preferably about 1×10^8 to about 1×10^{11}, and even more
preferably, about 1×10^9 to about 1×10^{10} activated CD8+

35   cells are utilized for adult humans, compared to about 5×10^6
- 5×10^7 cells used in mice.

            Preferably, as discussed above, the activated CD8+
cells are harvested from the cell culture prior to

administration of the CD8+ cells to the individual being
treated. It is important to note, however, that unlike other
present and proposed treatment modalities, the present method
uses a cell culture system that is not tumorigenic.
5   Therefore, if complete separation of stimulator cells and
activated CD8+ cells is not achieved, there is no inherent
danger known to be associated with the administration of a
small number of stimulator cells, whereas administration of
mammalian tumor-promoting cells may be extremely hazardous.
10   Methods of re-introducing cellular components are
known in the art and include procedures such as those
exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and
U.S. Patent No. 4,690,915 to Rosenberg. For example,
administration of activated CD8+ cells via intravenous
15   infusion is appropriate.
           The immunogenic peptides of this invention may also
be used to make monoclonal antibodies. Such antibodies may be
useful as potential diagnostic or therapeutic agents.
           The peptides may also find use as diagnostic
20   reagents. For example, a peptide of the invention may be used
to determine the susceptibility of a particular individual to
a treatment regimen which employs the peptide or related
peptides, and thus may be helpful in modifying an existing
treatment protocol or in determining a prognosis for an
25   affected individual. In addition, the peptides may also be
used to predict which individuals will be at substantial risk
for developing chronic infection.
           The following examples are offered by way of
illustration, not by way of limitation.
30

                              Example 1
                      Class I antigen isolation
           A flow diagram of an HLA-A antigen purification
scheme is presented in Figure 1. Briefly, the cells bearing
35   the appropriate allele were grown in large batches (6-8 liters
yielding ⁻5 x 10⁹ cells), harvested by centrifugation and
washed. All cell lines were maintained in RPMI 1640 media
(Sigma) supplemented with 10% fetal bovine serum (FBS) and

36

antibiotics. For large-scale cultures, cells were grown in roller bottle culture in RPMI 1640 with 10% FBS or with 10% horse serum and antibiotics. Cells were harvested by centrifugation at 1500 RPM IEC-CRU5000 centrifuge with 259

5   rotor and washed three times with phosphate-buffered saline (PBS)(0.01 M PO$_4$, 0.154 M NaCl, pH 7.2).

       Cells were pelleted and stored at -70°C or treated with detergent lysing solution to prepare detergent lysates. Cell lysates were prepared by the addition of stock detergent

10   solution [1% NP-40 (Sigma) or Renex 30 (Accurate Chem. Sci. Corp., Westbury, NY 11590), 150 mM NaCl, 50 mM Tris, pH 8.0] to the cell pellets (previously counted) at a ratio of 50-100 x 10^6 cells per ml detergent solution. A cocktail of protease inhibitors was added to the premeasured volume of stock

15   detergent solution immediately prior to the addition to the cell pellet. Addition of the protease inhibitor cocktail produced final concentrations of the following: phenylmethylsulfonyl fluoride (PMSF), 2 mM; aprotinin, 5 μg/ml; leupeptin, 10 μg/ml; pepstatin, 10 μg/ml;

20   iodoacetamide, 100 μM; and EDTA, 3 ng/ml. Cell lysis was allowed to proceed at 4°C for 1 hour with periodic mixing. Routinely 5-10 x 10^9 cells were lysed in 50-100 ml of detergent solution. The lysate was clarified by centrifugation at 15,000 x g for 30 minutes at 4°C and

25   subsequent passage of the supernatant fraction through a 0.2 μ filter unit (Nalgene).

       The HLA-A antigen purification was achieved using affinity columns prepared with mAb-conjugated Sepharose beads. For antibody production, cells were grown in RPMI with 10% FBS

30   in large tissue culture flasks (Corning 25160-225). Antibodies were purified from clarified tissue culture medium by ammonium sulfate fractionation followed by affinity chromatography on protein-A-Sepharose (Sigma). Briefly, saturated ammonium sulfate was added slowly with stirring to

35   the tissue culture supernatant to 45% (volume to volume) overnight at 4°C to precipitate the immunoglobulins. The precipitated proteins were harvested by centrifugation at 10,000 x g for 30 minutes. The precipitate was then dissolved

37

in a minimum volume of PBS and transferred to dialysis tubing (Spectro/Por 2, Mol. wt. cutoff 12,000-14,000, Spectum Medical Ind.). Dialysis was against PBS (≥20 times the protein solution volume) with 4-6 changes of dialysis buffer over a
5   24-48 hour period at 4°C. The dialyzed protein solution was clarified by centrifugation (10,000 x g for 30 minutes) and the pH of the solution adjusted to pH 8.0 with 1N NaOH. Protein-A-Sepharose (Sigma) was hydrated according to the manufacturer's instructions, and a protein-A-Sepharose column
10   was prepared. A column of 10 ml bed volume typically binds 50-100 mg of mouse IgG.

            The protein sample was loaded onto the protein-A-Sepharose column using a peristaltic pump for large loading volumes or by gravity for smaller volumes (<100 ml). The
15   column was washed with several volumes of PBS, and the eluate was monitored at A280 in a spectrophotometer until base line was reached. The bound antibody was eluted using 0.1 M citric acid at suitable pH (adjusted to the appropriate pH with IN NaOH). For mouse IgG-1 pH 6.5 was used for IgG2a pH 4.5 was
20   used and for IgG2b and IgG3 pH 3.0 was used. 2 M Tris base was used to neutralize the eluate. Fractions containing the antibody (monitored by A280) were pooled, dialyzed against PBS and further concentrated using an Amicon Stirred Cell system (Amicon Model 8050 with YM30 membrane). The anti-A2 mAb,
25   BB7.2, and the anti-A3 mAb, GAPA3, are particularly useful for affinity purification.

            The HLA-A antigen was purified using affinity columns prepared with mAb-conjugated Sepharose beads. The affinity columns were prepared by incubating protein-A-Sepharose beads
30   (Sigma) with affinity-purified mAb as described above. Five to 10 mg of mAb per ml of bead is the preferred ratio. The mAb bound beads were washed with borate buffer (borate buffer: 100 mM sodium tetraborate, 154 mM NaCl, pH 8.2) until the washes show A280 at based line. Dimethyl pimelimidate (20 mM)
35   in 200 mM triethanolamine was added to covalently crosslink the bound mAb to the protein-A-Sepharose (Schneider et al., _J. Biol. Chem._ 257:10766 (1982). After incubation for 45 minutes at room temperature on a rotator, the excess crosslinking

38

reagent was removed by washing the beads twice with 10-20 ml
of 20 mM ethanolamine, pH 8.2. Between each wash the slurry
was placed on a rotator for 5 minutes at room temperature.
The beads were washed with borate buffer and with PBS plus
5   0.02% sodium azide.

The cell lysate (5-10 x 10^9 cell equivalents) was
then slowly passed over a 5-10 ml affinity column (flow rate
of 0.1-0.25 ml per minute) to allow the binding of the antigen
to the immobilized antibody. After the lysate was allowed to
10   pass through the column, the column was washed sequentially
with 20 column volumes of detergent stock solution plus 0.1%
sodium dodecyl sulfate, 20 column volumes of 0.5 M NaCl, 20 mM
Tris, pH 8.0, and 10 column volumes of 20 mM Tris, pH 8.0.
The HLA-A antigen bound to the mAb was eluted with a basic
15   buffer solution (50 mM diethylamine in water). As an
alternative, acid solutions such as 0.15-0.25 M acetic acid
were also used to elute the bound antigen. An aliquot of the
eluate (1/50) was removed for protein quantification using
either a colorimetric assay (BCA assay, Pierce) or by SDS-
20   PAGE, or both. SDS-PAGE analysis was performed as described
by Laemmli (Laemmli, U.K., Nature 227:680 (1970)) using known
amounts of bovine serum albumin (Sigma) as a protein standard.

Allele specific antibodies were used to purify the
specific MHC molecule. In the case of HLA-A2 and HLA-A3 mAbs
25   BB7.2 and GAPA3 were used respectively. An example of SDS
PAGE analysis of purified HLA-A3.2 molecules is shown in
Figure 2.

Figure 2 shows SDS-PAGE (12.5%) analysis of affinity
purified HLA-A3.2 from the cell line EHM. An affinity column
30   (10 ml) was prepared with protein A-sepharose beads coupled to
the monoclonal antibody GAPA3 which is specific for HLA-A3. A
detergent lysate of 5x10^9 cells was passaged over the column
and the column was washed extensively. The bound HLA-A3.2
molecules were eluted from the column with 0.15M acetic acid,
35   50 ml. One ml of the eluate was removed and lyophilized to
concentrate the sample. The sample was taken up to 50 μl with
Leammli sample buffer and 20 μl were loaded in lane 2. Lane 1
contained molecular weight standards: Myosin, 230 kD; β-

galactosidase, 116kD; phosphorylase B, 97.4kD; bovine serum
albumin, 66.2kD; ovalbumin, 45kD; carbonic anhydrase, 31kD;
soybean trypsin inhibitor, 21.5kD; and lysozyme, 14.4kD.
Standard concentrations of bovine serum albumin were run in

5   lanes 8, 10 μg, 9, 3 μg, and 10, 1μg to aid in the estimation
of protein yield. For this particular HLA-A3.2 preparation,
the estimated yield was approximately 112 μg.

        For HLA-A11, A24.1 and A1, an alternative protocol
was used whereby anti-HLA-B and C monoclonal antibodies were

10   used to deplete HLA-B and C molecules. The remaining HLA-A
molecules were subsequently purified using the W6/32 mAb as
described below.

        Based on the density of class I expression as
indicated by the results of immunofluorescent staining

15   analysis, it is anticipated that average yields of class I
antigen isolated from the EBV B cell lines will range from
800-1200 μg per 10^{10} cell equivalents.

                        Example 2

20   An alternative class I purification protocol
        HLA-A2.1 molecules were isolated using the mAb
B1.23.2 which detects an epitope expressed by HLA-B and C
allele molecules, but not by HLA-A antigens. The mAb, W6/32,
detects all human class I molecules, including HLA-A, B and C.

25   As mentioned above, these mAbs react well with the B cell
lines serving as sources of HLA-A antigens. The B1.23.2 mAb
reacts with the various human B cell lines, but fails to react
with a mouse cell line that expresses a transfected HLA-A2.1
protein or a chimeric A2.1 mouse K^b molecule. It does react

30   with the human cell line, CIR (Alexander, J., et al.,
Immunogenetics, 29, 380 [1989]), that lacks expression of
HLA-A and B molecules, but expresses low levels of HLA-C
molecules. This pattern of reactivity illustrates how the
B1.23.2 mAb can be used to deplete the B cell lysates of HLA-B

35   and C molecules.

        Affinity columns were prepared using the affinity-
purified B1.23.2 and W6/32 mAbs, respectively, as described
above. The procedures for the preparation of the affinity

40

columns are essentially identical to the procedures described
for the preparation of the allele-specific mAb columns
described above. The B1.23.2 mAb affinity column was used to
deplete the detergent lysates of HLA-B and C molecules using
5   the protocol as described above. The cell lysate depleted of
HLA-B and C was then passed over a W6/32 mAb affinity column.
The MHC molecule that was eluted from this second passage was
the A allele product.

          This alternative affinity purification is useful for
10   the purification of any HLA-A allele product, and does not
rely on the need for allele-specific mAbs. In addition, it
could also be used to isolate any class I molecule type from
transfected cell lines.

15   Example 3

     Isolation and sequencing of naturally processed peptides

          For the HLA-A preparations derived from the base (50
mM diethylamine) elution protocol, the eluate was immediately
neutralized with 1 N acetic acid to pH 7.0-7.5. The
20   neutralized eluate was concentrated to a volume of 1-2 ml in
an Amicon stirred cell [Model 8050, with YM3 membranes
(Amicon)]. Ten ml of ammonium acetate (0.01 M, pH 8.0) was
added to the concentrator to remove the non-volatile salts,
and the sample was concentrated to approximately 1 ml. A
25   small sample (1/50) was removed for protein quantitation as
described above. The remainder was recovered into a 15 ml
polypropylene conical centrifuge tube (Falcon, 2097) (Becton
Dickinson). Glacial acetic acid was added to obtain a final
concentration of 10% acetic acid. The acidified sample was
30   placed in a boiling water bath for 5 minutes to allow for the
dissociation of the bound peptides. The sample was cooled on
ice, returned to the concentrator and the filtrate was
collected. Additional aliquots of 10% acetic acid (1-2 ml)
were added to the concentrator, and this filtrate was pooled
35   with the original filtrate. Finally, 1-2 ml of distilled
water was added to the concentrator, and this filtrate was
pooled as well.

The retentate contains the bulk of the HLA-A heavy chain and β_2-microglobulin, while the filtrate contains the naturally processed bound peptides and other components with molecular weights less than about 3000. The pooled filtrate material was lyophilized in order to concentrate the peptide fraction. The sample was then ready for further analysis.

For HPLC (high performance liquid chromatography) separation of the peptide fractions, the lyophilized sample was dissolved in 50 μl of distilled water, or into 0.1% trifluoroacetic acid (TFA) (Applied Biosystems) in water and injected to a C18 reverse-phase narrow bore column (Beckman C18 Ultrasphere, 10 x 250 mm), using a gradient system described by Stone and Williams (Stone, K.L. and Williams K.R., in, Macromolecular Sequencing and Synthesis; Selected Methods and Applications, A.R. Liss, New York, 1988, pp. 7-24. Buffer A was 0.06% TFA in water (Burdick-Jackson) and buffer B was 0.052% TFA in 80% acetonitrile (Burdick-Jackson). The flow rate was 0.250 ml/minute with the following gradient: 0-60 min., 2-37.5% B; 60-95 min., 37.5-75% B; 95-105 min., 75-98% B. The Gilson narrow bore HPLC configuration is particularly useful for this purpose, although other configurations work equally well.

A large number of peaks were detected by absorbance at 214 nm, many of which appear to be of low abundance (Fig. 3). Whether a given peak represents a single peptide or a peptide mixture was not determined. Pooled fractions were then sequenced to determine motifs specific for each allele as described below.

Pooled peptide fractions, prepared as described above were analyzed by automated Edman sequencing using the Applied Biosystems Model 477A automated sequencer. The sequencing method is based on the technique developed by Pehr Edman in the 1950s for the sequential degradation of proteins and peptides to determine the sequence of the constituent amino acids.

The protein or peptide to be sequenced was held by a 12-mm diameter porous glass fiber filter disk in a heated, argon-purged reaction chamber. The filter was generally pre-

42

treated with BioBrene Plus™ and then cycled through one or
more repetitions of the Edman reaction to reduce contaminants
and improve the efficiency of subsequent sample sequencing.
Following the pre-treatment of the filter, a solution of the
5   sample protein or peptide (10 pmol-5 nmol range) was loaded
onto the glass filter and dried. Thus, the sample was left
embedded in the film of the pre-treated disk. Covalent
attachment of the sample to the filter was usually not
necessary because the Edman chemistry utilized relatively
10   apolar solvents, in which proteins and peptides are poorly
soluble.

            Briefly, the Edman degradation reaction has three
steps: coupling, cleavage, and conversion. In coupling step,
phenylisothiocyanate (PITC) is added. The PITC reacts
15   quantitatively with the free amino-terminal amino acid of the
protein to form the phenylthiocarbamyl-protein in a basic
environment. After a period of time for the coupling step,
the excess chemicals are extracted and the highly volatile
organic acid, trifluoroacetic acid, TFA, is used to cleave the
20   PITC-coupled amino acid residue from the amino terminus of the
protein yielding the anilinothiazolinone (ATZ) derivative of
the amino acid. The remaining protein/peptide is left with a
new amino terminus and is ready for the next Edman cycle. The
ATZ amino acid is extracted and transferred to a conversion
25   flask, where upon addition of 25% TFA in water, the ATZ amino
acid is converted to the more stable phenylthiohydantoin (PTH)
amino acid that can be identified and quantified following
automatic injection into the Model 120 PTH Analyzer which uses
a microbore C-18 reverse-phase HPLC column for the analysis.
30   In the present procedures, peptide mixtures were
loaded onto the glass filters. Thus, a single amino acid
sequence usually does not result. Rather, mixtures of amino
acids in different yield are found. When the particular
residue is conserved among the peptides being sequenced,
35   increased yield for that amino acid is observed.

                         Example 4
                Definition of an A3.2 specific motif

43

There is some ambiguity in the international nomenclature of A3 alleles. The A3.2 allele herein is expressed by cell lines EHM, HO301, and GM3107. This particular subtype is currently referred to as the 3.2 allele

5   (Yang, in _Immunobiology of HLA_, Vol. 1, Dupont ed., Springer-Verlag, New York pp. 43-44 and 54-55, 1989), or the product of the A*0301 gene (its sequence corresponds to the one published by Strachan, et al., _EMBO J._, 3:887 (1984), and has been verified by direct cloning and sequencing of the A3 gene found

10   in EHM cell line. The HLA-A3.2 encoded by the A*0301 gene referred to in this document is the commonly expressed HLA-A3 allelic form.

In one case using MAT cells, pooled peptide fractions prepared as described in Example 3 above were obtained from

15   HLA-A3.2 homozygous cell lines, for example, CM3107. The pooled fractions were HPLC fractions corresponding to 7% to 19% CH_3CN. For this class I molecule, this region of the chromatogram was most abundant in peptides. Data from independent experiments were averaged as described below.

20   The amino acid sequence analyses from four independent experiments were analyzed and the results are shown in Table 5. For each position except the first, the data were analyzed by modifying the method described by Falk et al. to allow for comparison of experiments from different

25   HLA types. This modified procedure yielded quantitative yet standardized values while allowing the averaging of data from different experiments involving the same HLA type.

The raw sequenator data was converted to a simple matrix of 10 rows (each representing one Edman degradation

30   cycle) and 16 columns (each representing one of the twenty amino acids; W, C, R and H were eliminated for technical reasons. The data corresponding to the first row (first cycle) was not considered further because, this cycle is usually heavily contaminated by free amino acids.). The

35   values of each row were summed to yield a total pmoles value for that particular cycle. For each row, values for each amino acid were then divided by the corresponding total yield value, to determine what fraction of the total signal is

44

attributable to each amino acid at each cycle. By doing so,
an "Absolute Frequency" table was generated. This absolute
frequency table allows correction for the declining yields of
each cycle.

5   Starting from the absolute frequency table, a
"relative frequency" table was then generated to allow
comparisons among different amino acids. To do so the data
from each column was summed, and then averaged. Then, each
value was divided next by the average column value to obtain
10   relative frequency values. These values quantitate, in a
standardized manner, increases and decreases per cycle, for
each of the different sixteen amino acid types. Tables
generated from data from different experiments can thus be
added together to generate average relative frequency values
15   (and their standard deviations). All standard deviations can
then be averaged, to estimate a standard deviation value
applicable to the samples from each table. Any particular
value exceeding 1.00 by more than two standard deviations is
considered to correspond to a significant increase.

20   The results of the foregoing analysis for HLA-A3.2
were as follows: at position 2, a 2.2-fold increase in valine
(V) with lesser increases (1.5-1.7) for structurally similar
residues leucine (L) and methionine (M). At position 3,
tyrosine (Y) and aspartic acid (D) showed increases in
25   frequency. At position 7 isoleucine (I) was increased, and at
position 8 asparagine (N) and glutamine (Q) were increased.
At positions 9 and 10, lysine (K) was increased more than 2-
fold over the expected random yield.

          Cysteine was not modified and thus not detected.
30   PTH-tryptophan coeluted with diphenylurea, and in some
experiments, PTH-arginine coeluted with the major derivative
of PTH-threonine. Therefore, cysteine and tryptophan are not
detectable and arginine is detected only in the absence of
threonine.

35   Previously described MHC structures showed instances
of critically conserved residues at position 2 (or 3) and at
the C terminus (either position 9 or 10). These residues are
referred to as "conserved" residues. The modified data

45

analysis of this invention considered the conserved positions
at the N and C terminals.

  Thus, the HLA-A3.2 motif should have position two
occupied by V, L or M, a length of 9 or 10 amino acids, and a
5 C-terminal position occupied by K.

TABLE 5
Summary
HLA-A3.2 Allele-Specific Motif

10

Position	Conserved Residues
1	-
2	V,L,M
3	Y,D
4	-
5	-
6	-
7	I
8	Q,N
9	K
10	K

Example 5

25 Definition of HLA-A1-specific peptide motifs

  HLA-A1 molecules were isolated and their naturally
processed peptides characterized, as described in Example 3
above. In one case using MAT cells, pooled fractions
corresponding to 19% to 50% CH₃CN were used. As in the
30 preceding example, residues showing at any given position
except the first position, at least a two standard deviation
increase over the random expected yield were identified and
shown in Table 6. On the basis of these data, only Serine (S)
and Threonine (T) were increased at position two. At position
35 3, aspartic acid (D) and glutamic acid (E) were elevated and
at position 9 and 10 tyrosine (Y) showed a marked increase.
Other increases noted were proline (P) at position 4 and
leucine (L) at position 7. Therefore, the motifs for HLA-A1
based on these data would have residues at position 2 occupied
40 by S or T, a peptide length of 9 or 10 amino acids and a

46

C-terminal residue of Y. Alternatively, another motif would
comprise a D or E at position 3 together with a C terminal
residue of Y.

TABLE 6
Summary
HLA-A1 Allele-Specific Motif

Position	Conserved Residues
1	-
2	S, T
3	D,E
4	P
5	-
6	-
7	L
8	-
9	Y

Example 6

Definition of HLA-A11 allele-specific peptide motifs

HLA-A11 motifs were defined by amino acid sequence
analysis of pooled HPLC fractions, in one case corresponding
to 7% to 45% CH₃CN of fractionated peptides eluted from
HLA-A11 molecules purified from the cell line BVR. On the
basis of the data presented in Table 7, a motif for A11
consists of a conserved residue at position 2 of threonine (T)
or valine (V), a peptide length of 9 or 10 amino acids, and a
C-terminal conserved residue of lysine (K). At position 3
increases in methionine (M) and phenylalanine (F) were also
seen and at position 8 glutamine (Q) was increased.

TABLE 7
Summary
HLA-A11 Allele-Specific Motif

Position	Conserved Residues
1	-
2	T,V
3	M,F
4	-

47

5	-
6	-
7	-
8	Q
9	K
10	K

Example 7

Definition of HLA-A24.1 Specific Peptide Motifs

HLA-A24.1 allele-specific motifs were defined by amino acid sequence analysis of pooled fractions in one case corresponding to 7% to 19% CH_3CN of HPLC fractionated peptides eluted from HLA-A24.1 molecules purified from the cell line KT3. On the basis of the data presented in Table 8 a motif for HLA-A24.1 consists of a conserved residue at position 2 occupied by tyrosine (Y), a peptide length of 9 or 10 amino acids, and a C-terminal conserved residue of phenylalanine (F) or leucine (L). Increases were also observed at several other positions: isoleucine (I) and methonine (M) at position 3; aspartic acid (D), glutamic acid (E), glycine (G), lysine (K) and proline (P) at position 4; lysine (K), methonine (M) and asparagine (N) at position 5; valine (V) at position 6; asparagine (N) and valine (V) at position 7; and, alanine (A), glutamic acid (E), lysine (K), glutamine (Q) and serine (S) at position 8.

Table 8
Summary
HLA-A24.1 Allele-Specific Motif

Position	Conserved Residues
1	-
2	Y
3	I,M
4	D,E,G,K,P
5	L,M,N
6	V
7	N,V

8	A,E,K,Q,S
9	F,L
10	F,A

Example 8

Identification of immunogenic peptides

Using the motifs identified above for various MHC class I allele amino acid sequences from various viral and tumor-related proteins were analyzed for the presence of these motifs. Sequences for all of the target antigens were obtained from the GenBank data base (Release No. 71.0; 3/92). The identification of motifs was done using the "FINDPATTERNS" program (Devereux, Haeberli and Smithes (1984). Nucleic Acids Research 12(1); 387-395).

The amino acid sequence or the nucleotide sequence encoding products was obtained from the GenBank database. In the cases of Human Papilloma Virus (HPV), Prostate Specific antigen (PSA), p53 oncogene, Epstein Barr Nuclear Antigen-1 (EBNA-1), and c-erb2 oncogene (also called HER-2/neu), and Melanoma Antigen-1 (MAGE-1), a single sequence exists.

In the cases of Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV) several strains/isolates exist and many sequences have been placed in GenBank.

For HBV, binding motifs were identified for the adr, adw and ayw types. In order to avoid replication of identical sequences, all of the adr motifs and only those motifs from adw and ayw that are not present in adr were added to the list of peptides.

In the case of HCV, a consensus sequence from residue 1 to residue 782 was derived from 9 viral isolates. Motifs were identified on those regions that had no or very little (one residue) variation between the 9 isolates. The sequences of residues 783 to 3010 from 5 viral isolates were also analyzed. Motifs common to all the isolates were identified and added to the peptide list.

Finally, a consensus sequence for HIV type 1 for North American viral isolates (10-12 viruses) was obtained

from the Los Alamos National Laboratory database (May 1991
release) and analyzed in order to identify motifs that are
constant throughout most viral isolates. Motifs that bear a
small degree of variation (one residue, in 2 forms) were also
5   added to the peptide list.

        Several motifs for each allele shown below were used
to screen several antigens. Protein E6 of human papilloma
virus (HPV) type 16 using motifs from all of the alleles
disclosed above are shown (Table 9). Protein E7 of HPV type
10 18 was also searched for motifs from all alleles (Table 9)
Melanoma antigens MAGE 1, 2 and 3 were searched for motifs
from all alleles (Table 10). The antigen PSA was searched for
motifs from all alleles (Table 11). Finally, core and
envelope proteins from hepatitis C virus were also searched
15 (Table 12). In the tables and the description of the motifs,
the conventional symbol letter for each amino acid was used.
The letter "X" represents a wild card character (any amino
acid).

        The following motifs were screened in the present
20 search:

            For HLA-A1 (A*0101):
            1   XSXXXXXXY
            2   XSXXXXXXXY
25   3   XTXXXXXXY
            4   XTXXXXXXXY
            5   XXDXXXXXY
            6   XXDXXXXXXY
            7   XXEXXXXXY
30   8   XXEXXXXXXY

            For HLA-A3.2 (A*0301)
            1   XVXXXXXXK
            2   XVXXXXXXXK
35   3   XLXXXXXXK
            4   XLXXXXXXXK
            5   XMXXXXXXK
            6   XMXXXXXXXK

50

For HLA-A11 (A*1101):

1   XTXXXXXXK
2   XTXXXXXXXK
3   XVXXXXXXK
4   XVXXXXXXXK

For HLA-A24.1 (A*2401):

1   XYXXXXXXF
2   XYXXXXXXXF
3   XYXXXXXXL
4   XYXXXXXXXL

Peptides with MHC Class I Binding Motifs   Table 9

51

AA Position	Sequence	Antigen	HLA molecule
	30 IHDIILECVY	HPV16.E6	A1
	69 VCDKCLKFY	HPV16.E6	A1
5	77 YSKISEYRHY	HPV16.E6	A1
	80 ISEYRHYCY	HPV16.E6	A1
	92 GTTLEQQYNK	HPV16.E6	A11
	93 TTLEQQYNK	HPV16.E6	A11
	106 LLIRCINCQK	HPV16.E6	A3
10			
	2 HGDTPTLHEY	HPV16.E7	A1
	16 QPETTDLYCY	HPV16.E7	A1
	44 QAEPDRAHY	HPV16.E7	A1
	89 IVCPICSQK	HPV16.E7	A3, A11
15			
	3 RFEDPTRRPY	HPV18.E6	A1
	4 FEDPTRRPY	HPV18.E6	A1
	25 LQDIEITCVY	HPV18.E6	A1
	41 LTEVFEFAFK	HPV18.E6	A11
20	72 YSRIRELRHY	HPV18.E6	A1
	84 SVYGDTLEK	HPV18.E6	A3, A11
	101 LLIRCLRCQK	HPV18.E6	A3
	59 HTMLCMCCK	HPV18.E7	A11
25			

Human Papilloma Virus 16 and 18 (E6 and E7 Proteins)

Peptides with MHC Class I Binding Motifs   Table 10

AA Position	Sequence	Antigen	HLA molecule
2 SLEQRSLHCK		MAGE 1	A3
96 SLFRAVITK		MAGE 1	A3
96 SLFRAVITKK		MAGE 1	A3
108 DLVGFLLLK		MAGE 1	A3
128 MLESVIKNYK		MAGE 1	A3
128 MLESVIKNY		MAGE 1	A1
152 QLVFGIDVK		MAGE 1	A3
161 EADPTGHSY		MAGE 1	A1
182 LLGDNQIMPK		MAGE 1	A3
215 WEELSVMEVY		MAGE 1	A1
223 VYDGREHSAY		MAGE 1	A1
238 LLTQDLVQEK		MAGE 1	A3
239 LTQDLVQEK		MAGE 1	A11
239 LTQDLVQEKY		MAGE 1	A1
240 TQDLVQEKY		MAGE 1	A1

Melanoma Antigen MAGE 1

Peptides with MHC Class I Binding Motifs   Table 11

AA Position	Sequence	Antigen	HLA molecule
21	IVGGWECEK	PSA	A3, A11
57	LTAAHCIRNK	PSA	A11
88	VSHSFPHPLY	PSA	A1
95	PLYDMSLLK	PSA	A3
178	DVCAQVHPQK	PSA	A3, A11
182	QVHPQKVTK	PSA	A3, A11
236	PSLYTKVVHY	PSA	A1
239	YTKVVHYRK	PSA	A11
241	KVVHYRKWIK	PSA	A3, A11
242	VVHYRKWIK	PSA	A3, A11

Prostate Specific Antigen (PSA)

54

Peptides with MHC Class I Binding Motifs   Table 12

AA Position	Sequence	Antigen	HLA molecule
2	STNPKPQRK	HCV	A11
14	NTNRRPQDVK	HCV	A11
43	RLGVRATRK	HCV	A3
302	VQDCNCSIY	HCV	A1
556	WMNSTGFTK	HCV	A3
605	LTPRCMVDY	HCV	A1
626	FTIFKIRMY	HCV	A1

Hepatitis C Virus (Consensus Sequence)

55

Example 9

Quantitative HLA class I binding assay

To verify that motif-containing peptide sequences are indeed capable of binding to the appropriate class I
5   molecules, specific binding assays were established. HLA-A3.2 molecules were purified from GM3107 EBV cells by affinity chromatography using the GAPA3 mAb (anti-A3) to isolate A3.2. Prior to the step, the lysate was depleted of HLA B and C molecules by repeated passages over a B1.23.2 column (this
10   antibody is B,C specific) generally as described in Example 2, above.

As a radiolabeled probe, the peptide 941.12 (KVFPYALINK), containing an A3.2 motif, was used. This peptide contains the anchor residues V_2 and K_{10}, associated
15   with A3.2-specific binders, described above. A Y residue was inserted at position 5 to allow for radiolodination. Peptides were labeled by the use of the Chloramine T method Buus et al., Science 235:1352 (1987), which is incorporated herein by reference.

20   A dose range of purified A3.2 was incubated with 10 nM of 941.12 at pH 7.0 and 23°C, in presence of a protease inhibitor cocktail (1 mM PMSF, 1.3 mM 1.10 phenanthroline, 73 µM pepstatin A, 8 mM EDTA, and 200 µM N a_p-tosyl-L-lysine chloromethyl ketone (TLCK)), in presence of 1 µM purified
25   human β2 microglobulin. After two days, the % bound radioactivity was measured by gel filtration over TSK 2000 columns as previously described for class II peptide binding assays in Sette et al., in Seminars in Immunology, Vol. 3, Gefter, ed. (W.B. Saunders, Philadelphia, 1991), pp 195-202,
30   which is incorporated herein by reference. (see, Fig. 4). Good binding (in the 60 to 100% range) was observed for A3.2 concentrations ranging between 35 and 300 nM. 30% binding was observed at 15 nM A3.2.

To minimize A3.2 usage and to increase the
35   sensitivity of the assay, a concentration of 5-10 nM A3.2 was selected for further assays. In the experiment shown in Fig. 5, 7nM A3.2 and an equivalent concentration of radiolabeled 941.12 were incubated using the conditions described above and

56

in the presence of a dose range of three peptides (HBc 18-27
(924.07), a Prostate Specific Antigen peptide (939.01), and
HIV nef 73-82 (940.03)). It was found that peptide 940.03
inhibited strongly, with a 50% inhibitory concentration

5   (IC50%) of 22 nM, while a weaker inhibition was observed with
peptide 939.01 (IC50% 940 nM). Finally, peptide 924.07 did
not show any inhibition up to the 30 μM level. Thus, it is
concluded that peptides 940.03 and 939.01 are high and
intermediate affinity binders, respectively, while peptide

10   924.07 is classified as a low affinity or negative binder.
          Throughout this disclosure, results have been
expressed in terms of IC50's. Given the conditions in which
the assays are run (i.e., limiting MHC and labeled peptide
concentrations), these values approximate K_D values. It

15   should be noted that IC50 values can change, often
dramatically, if the assay conditions are varied, and
depending on the particular reagents used (e.g., Class I
preparation, etc.). For example, excessive concentrations of
MHC will increase the apparent measured IC50 of a given

20   ligand.
          An alternative way of expressing the binding data, to
avoid these uncertainties, is as a relative value to a
reference peptide. The reference peptide is included in every
assay. As a particular assay becomes more, or less,

25   sensitive, the IC50's of the peptides tested may change
somewhat. However, the binding relative to the reference
peptide will not change. For example, in an assay run under
conditions such that the IC50 of the reference peptide
increases 10-fold, all IC50 values will also shift

30   approximately 10-fold. Therefore, to avoid ambiguities, the
assessment of whether a peptide is a good, intermediate, weak,
or negative binder should be based on it's IC50, relative to
the IC50 of the standard peptide.
          If the IC50 of the standard peptide measured in a

35   particular assay is different from that reported in the table,
then it should be understood that the threshold values used to
determine good, intermediate, weak, and negative binders
should be modified by a corresponding factor. For example, if

57

in an A2.1 binding assay, the IC50 of the A2.1 standard
(941.01) were to be measured as 8 nM instead of 5 nM, then a
peptide ligand would be called a good binder only if it had an
IC50 of less than 80 nM (i.e., 8nM x 0.1), instead of the
usual cut-off value of 50 nM.

The experimental system herein described can be used
to test binding of large numbers of synthetic peptides to a
variety of different class I specificities. Specific binding
assays can be performed as follows.

HLA-A11-specific assay

The cell line BVR was used as a source of HLA. The
dependency of the binding on MHC concentration in presence or
absence of β_2M are shown in Fig. 6, while Fig. 7 depicts the
dose dependency of the inhibition by excess unlabeled ligand.
Finally, Fig. 8 shows a Scatchard analysis experiment. Values
of apparent kD of ~6 nM and of 10% active receptor were
obtained, and were remarkable for their similarity to the
values obtained for A2.1 and A3.2. The sequence of the peptide
used as a radiolabeled probe (940-06) is AVDLYHFLK.

HLA-A1-specific assay

In this case, the EBV cell line Steinlin was used as
a source of purified HLA. The same protocol previously
applied to purification of other HLA alleles (i.e., depletion
of B, C molecules by a B1.23.2 mAb column, followed by
purification of A molecules by means of a W632 mAb column) was
utilized. On the basis of the pool sequencing data, consensus
peptides were synthesized, directly radiolabeled, and tested
for HLA binding using the standard protocol (1 mM β_2M, 2 days
RT incubation in presence of protease inhibitors). A graph
illustrating the relationship between % binding and μM input
HLA A1 is shown in Fig. 9. From the data, it was concluded
that in analogy with what was observed for HLA A2, 3, and 11,
as little as 30 nM are sufficient to obtain ~10% binding. The
sequence of the peptide used as a radiolabeled probe (944.02)
is YLEPAIAKY. In the next set of experiments, the specificity
of the assay established was verified by its inhabitability by
excess unlabeled peptide. The IC50% was measured (Fig. 10) as

58

~20 nM. Further Scatchard analysis (Fig. 11) verified that
the apparent K_D of the interaction corresponded to 21 nM, with
a % of active receptor corresponding to 5.1%.

5   HLA-A24 specific assay

          HLA A24 molecules were purified from the KT3 EBV cell
line. In this case, two consensus peptides whose sequences
were based on the pool sequencing data have been synthesized.
Their sequences are: 979-01, AYIDNVYKF and 979.02, AYIDNYNKF.
10   The results of experiments in which the % bound of these two
peptides as a function of input MHC was measured are shown in
Fig. 12. In both cases, 10-15% binding was obtained with as
little as 20-50 nM MHC. Cold inhibition experiments (Fig.
13), limiting MHC concentrations, revealed that the binding
15   was readily inhibitable by excess unlabeled peptide, with an
apparent K_D of 30 and 60 nM, respectively. Further Scatchard
experiments verified values of 136 nM and 28 nM, respectively.
The apparent % of available receptor (active MHC) were 8.3%
and 7.4%, respectively (Fig. 9a and b). On the basis of these
20   data, peptide 979.02 was arbitrarily selected as standard
label indicator for A24 assays. Furthermore, on the basis of
the data herein described, we also conclude that the goal of
establishing an A24-specific binding assay has been
accomplished. In conclusion, specific assays for the five
25   major HLA alleles have been described.

                          Example 10
                    Expansion of HLA A Motifs

          Establishing *in vitro* binding assays allows one to
30   readily quantitate *in vitro* the binding capacity of various
synthetic peptides to the various alleles of interest (HLA A1,
A2, A3, A11, and A24). This allows verification of the
correctness of the motifs by means of peptides carrying the
various HLA A motifs for their capacity to bind purified HLA
35   molecules. Typically, peptides were synthesized with specific
HLA motifs embedded in a neutral backbone composed of only
alanine residues. In some cases, a K residue was also
introduced within the sequence, with the purpose of increasing

59

solubility. The use of such "neutral" poly A backbones, as
applied to the case of class II molecules, has been described
in detail, for example, by Jardetzky et al. (Jardetzky et al.,
EMBO J. 9(6):1797,1990).

For example, in the case of A3.2, a motif has been
defined with a hydrophobic residue in position 2 and a
positive charge (K) in position 9. Thus, to verify that the
presence of these two anchor residues would allow, in the
context of a poly A backbone, for A3.2 binding, the poly A
analog with the sequence AMAAAAAAK was synthesized (Table 13).

Similarly, other peptides carrying other HLA motifs
were also synthesized and tested for HLA binding. It was
found that in all cases, the presence of the specific HLA
motifs was conducive to binding to the relevant HLA allele,
with estimated K_D comprised of between 125 and 2.8 nM. In
most cases, the binding was also absolutely specific, in that
no binding was detected to irrelevant alleles. Only two
exceptions to this general rule were observed. Firstly, A3
and A11 peptides crossreacted extensively with each other,
perhaps as could have been expected by the fact that the
motifs for these two alleles are remarkably similar. Second,
some A1 peptides crossreacted, albeit with much lower
affinities, on A11 and A3.2.

To further define the structural requirements for the
interaction between peptide epitopes and various class I
alleles of interest, analogs of 10 residues in length of some
of the 9 residue peptides shown in Table 13 were synthesized
(Table 14). These analogs were generated by inserting an
additional Ala residue within the poly A backbone, so that the
anchor residues are not located in positions 2 and 10 (as
opposed to 2 and 9 in the previous Table). The results
obtained illustrate that motifs of 10 residues are also
capable of specifically binding to the relevant class I
alleles, albeit with a slightly lower efficiency.

In summary, these data confirm that both 9-mer and
10-mer peptides which contain the appropriate motifs can bind
HLA. On the basis of these data, 8-mer or 11-mer peptides

should also be capable of binding, even if perhaps with lower
affinities.

The data described above show that the presence of
certain residues in the anchor positions does allow (at least

5   in a "neutral" poly A backbone) for HLA binding. To
investigate to what degree other amino acids (for example,
chemically related amino acids) might be tolerated in these
crucial anchor positions, analogs of some of the poly A
peptides from Table 13 were synthesized, in which the residue

10   present in position 2 (or 3) or 9 was varied. The results of
this analysis are shown in Tables 15-19.

In the case of A3.2 (Table 15), in position 2, L, M,
I, V, S, A, T, and F were found to be preferred (binding ≥ 0.1
relative to previously defined anchor residues), while C, G,

15   and D were permitted (binding ≥ 0.01 to 0.1 relative to
previously defined anchor residues). The substitution of E,
because of its similarity to D, in this position should also
be tolerated. In position 9, K, R, and Y were preferred.
Because of a similarity in nature, that H and F should also be

20   preferred. No other residue was tolerated in position 9 for
A3 binding.

In the case of A11 (Table 16), the preferred residues
in position 2 were L, M, I, V, A, S, T, G, N (L and Q by
similarity). Tolerated were C, F, D (and E by similarity).

25   In position 9, K was preferred and R was tolerated. H should
also be tolerated by similarity.

In the case of A24 (Table 17), Y and F were preferred
in position 2 (and W by similarity); no other residue was
tolerated. In position 9, F, I, and L were preferred (and W

30   and M by extension). No other residue was tolerated.

In the case of A1, three different anchor residues
had previously been defined. The results shown in the
preceding section show that they act independently of each
other (i.e., that two out of three anchors would be sufficient

35   for binding). This is indeed the case. For this reason,
analogs containing two anchors were synthesized to define what
residues might be preferred or tolerated in each position.
The data shown in Table 18 show that in position 2, T, S, and

M are preferred, and no other residue is tolerated. In
position 3 (Table 19), D and E are preferred, and A,S (and T
by similarity) are tolerated. Finally, in position 9, only Y
is preferred, and no other residue appears to be tolerated
5   (Table 19).

        Thus, on the basis of the data, it is concluded that
peptides carrying any combination of two preferred residues
can bind.   Peptides containing "imperfect" motifs, i.e.,
carrying a preferred residue at one position and a tolerated
10   one at the other anchor position, should also be capable of
binding, even if with somewhat lower affinity. Using the
motifs of this invention for various MHC class I alleles amino
acid sequences from various viral and tumor-related proteins
were analyzed for the presence of motifs. The results of this
15   motif analysis is shown in Table 23 a - k.

                        Example 11
    Validation of HLA Peptide Binding Motifs with an Unbiased Set
                       of HPV 16 Peptides.
20   Human Papillomaviruses (HPVs) are implicated in the
etiology of cervical cancer (Pfister, H. (1974) Biology and
biochemistry of papillomaviruses, Rev. Physiol. Biochem.
99:111; zur Hausen, H. (1991). Human papillomaviruses in the
pathogenesis of anogenital cancer. Virology. 184:9) and in up
25   to 10% of total mortality due to cancer worldwide (zur Hausen,
H. (1991). Viruses in Human Cancers. Science, 254:1167).
Cervical cancer is the second most common cause of
cancer-related death in females worldwide (Parkin, D. M.,
Laara, E., and Muir, C. S. (1988), Estimates of the worldwide
30   frequency of sixteen major cancers in (1980). Int. J. Cancer.
41:184). HPV DNA is present in more than 90% of the cervical
carcinomas and predominantly of the HPV 16 genotype (Resnick,
R. M., Cornelissen, M. T., Wright, D, K., Eichinger, G. H.,
Fox, H. S., ter Schegget, J., and Manos, M. M. (1990).
35   Detection and typing of human papillomavirus in archival
cervical cancer specimens by DNA amplification with consensus
primers. J. Natl. Cancer Inst; Van den Brule, A. J. C.,
Walboomers, J. M. M., du Maine, M., Kenemans, P., and Meijer,

C. J. L. M. (1991). Difference in prevalence of human
papillomavirus genotypes in cytomorphologically normal smears
is associated with a history of cervical intraepithetal
neoplasia. *Int. J. Cancer.* 48:404). The ability of HPV 16
early region 6 and 7 (E6, E7) open reading frames to in vitro
immortalize rodent cells (Yasumoto, S., Burkhardt, A.L.,
Doniger, J., and DiPaolo, J.A. (1986). Human Papillomaviruses
type 16 DNA induced malignant transformation of NIH3T3 cells.
J. Virol. 57:572) and human keratinocytes (Pirisi. L.,
Yasumoto, S., Feller, M., Doniger, J., and DiPaolo, J.A.
(1987). Trans-formation of human fibroblasts and
keratinocytes with human papillomavirus type 16 DNA. *J.
Virol,* 61:1061) and to transform human fibroblasts (Smits, H.
L., Raadsheer, E., Rood, I., Mehendale, S., Slater, R. M., van
der Noordaa, J., and ter Schegget, J. (1988). Induction of
anchorage-independent growth of human embryonic fibroblasts
with a deletion in the short arm of chromosome 11. *J. Virol.*
62:4538) suggests direct involvement of HPV 16 in the
multi-step process of cervical carcinogenesis,

     In general T cell immunity, in particular mediated by
cytotoxic T lymphocytes (CTL) is important in the defense
against virus-induced tumors (Melief, C. J. (1992). Tumor
eradication by adoptive transfer of cytotoxic T lymphocytes.
Adv. Cancer Res. 58:143; Melief, C. J., and Kast, W. M.
(1992). Lessons from T cell responses to virus induced tumors
for cancer eradication in general. *Cancer Surv.* 13:81).
Recently in a mouse model, it was reported that some degree of
protection against HPV 16 E7 expressing tumors can be obtained
with CTL after immunization with HPV 16 E7 expressing cells
(Chen. L., Thomas, E, K., Hu, S. L., Hellström, I., and
Hellstrom, K. E. (1991). Human papillomavirus type 16
nucleoprotein E7 is a tumor rejection antigen. *Proc. Natl.
Acad. Sci.* 88:110; Chen, L., Ashe, S., Brady, W. A.,
Hellstrom, I., Hellström, K. E., Ledbetter, J. A,, McGowan,
P., and Linsley, P. S. (1992). Costimulation of Antitumor
immunity by the B7 counterreceptor for the T lymphocyte
molecules CD28 and CTLA-4. *Cell.* 71:1093). In vivo
protection by CTL was recently shown in mouse models in which

63

synthetic peptides containing CTL epitopes were used for
efficient priming of mice against virus infections (Schulz,
M., Zinkernagel, R. M., and Hengarter, H. (1991).
Peptide-induced antiviral protection by cytotoxic T cells.
Proc. Natl. Acad. Sci. USA 88:991; Kast, W. M., Roux, L.,
Curren, J., Blom, H. J. J., Voordouw, A. C., Meleon, R. H.,
Kolakofski, D., and Melief, C. J. M. (1991). Protection
against lethal Sendai virus infection by in vivo priming of
virus-specific cytotoxic T lymphocytes with an unbound
peptide. *Proc. Natl. Acad. Sci. USA.* 88:2283). Moreover in a
mouse model it has now been shown that complete protection
against HPV 16 induced tumors can be achieved by peptide
vaccination with a CTL epitope derived from the viral oncogene
E7().

        The HPV 16 E6 and E7 gene products are the most
desirable target antigens for vaccination against HPV 16
induced tumors. Both are retained and highly expressed in HPV
16-transformed cancer cells in vivo (Baker, C. J., Phelps, W.
C., Lindgren, V., Braun, M. J., Gonda, M. A., and Howley, P.
M. [1987]. Structural and transcriptional analysis of human
papillomavirus type 16 sequences in cervical carcinoma cell
lines. *J. Virol.* 61:962; Smotkin, D., and Wettstein, F. O.
[1986]. Transcription of human papillomavirus type 16 early
genes in a cervical cancer and cancer-derived cell line and
identification of the E7 protein. *Proc. Natl. Acad. Sci. USA.*
83:4680) and involved in the induction and maintenance of
cellular transformation in vitro (Crook, T., Morgenstern, J.
P., Crawford, L., and Banks, L. [1989]. Continued expression
of HPV-16 E7 protein is required for maintenance of the
transformed phenotype of cells co-transformed by HPV-16 plus
EJ ras. *EMBO J.* 8:513; Hawley-Nelson, P., Vousden, K. H.,
Hubbert, N. L., Lowy, D. R., and Schiller, J. T. [1989]. HPV
16 E6 and E7 proteins cooperate to immortalize human foreskin
keratinocytes. *EMBO J.* 8:3905). Dependence of in vitro growth
of cell lines derived from cervical cancers on the expression
of E6 and E7 emphasizes involvement of these oncogenes in
maintenance of the phenotype of cervical carcinoma cell lines
(Von Knebel Doeberitz, M., Bauknect, T., Bartch, D., and zur

64

Hausen, H. [1991]. Influence of chromosomal integration on
glucocorticoid-regulated transcription of growth-stimulation
papillomavirus genes E6 and E7 in cervical carcinoma cells.
Proc. Natl. Acad. Sci. USA. 88:1411). To determine the CTL
epitopes and potential vaccine candidates of HPV 16 for
humans, we screened peptides spanning the HPV 16 E6 and E7
protein sequences for their ability to bind to the most
frequent human MHC molecules, namely HLA-A1, A3.2, A11.2 and
A24. Combined these five alleles will cover about 90% of the
world population (Dupont, B., ed. [1987]. Immunology of HLA
Vol. I -- Histocompatibility Testing. Springer-Verlag, New
York).

          A complete set of 240 overlapping synthetic peptides
of 9 aa length and 8 aa overlap covering the entire HPV 16 E6
and E7 oncogene sequences were synthesized. The peptides were
tested for their ability to bind the aforementioned HLA
molecules in the binding assay described above. The results
of this analysis show the relative affinity of all peptides
for the respective HLA alleles and reveal the possible
candidate CTL epitopes for use in peptide based vaccines for
humans in Tables 20(a)-(d).

          The results confirm that peptide binding motif
described in this invention for the aforementioned HLA alleles
predict which peptide of a protein is likely to bind into the
groove of a specified HLA molecule. Since we used a large and
unbiased set of peptides, the results of the peptide binding
analyses were used to evaluate the value of these motifs both
for their predictive capacities and the necessity to have
particular anchor aa residues on positions 2, (3) and 9 in a
peptide.

          Peptides. Peptides were generated by solid phase
strategies on a multiple peptide synthesizer (Abimed AMS 422)
by repeated cycles in which addition of Fmoc protected amino
acids to a resin of polystyrene was alternated with a
Fmoc-deprotection procedure (Gausepohl, H., Kraft, M., Boulin,
Ch., and Frank, R. W. [1990]. Automated multiple peptide
synthesis with BOP activation. *in* Proc. of the 11th American
peptide symposium. J. E. Rivier and G. R. Marshall, Ed.

ESCOM, Leiden. 1003-1004). The peptides all carrying a COOH
group at the C-terminal end, were cleaved from the resin and
side chain protective groups were removed by treatment with
aqueous TFA. Peptides were analyzed by reversed phase HPLC

5   lyophilized and dissolved at a concentration of 1 mg/ml in
phosphate-buffered saline with 3% DMSO (Sigma, St. Louis, MO
63175) before use. Once dissolved, the peptides were stored
at -70° C. Since cysteine containing peptides are susceptible
to (air) oxidation during synthesis and handling, these

10   peptides were synthesized with an alanine instead of a
cysteine.

        *Identification of peptides from HPV 16 E6 and E7
proteins that bind to different HLA-A alleles.* A complete set
of 240 peptides of 9 aa in length and overlapping by 8 aa,

15   covering the sequences of the entire HPV 16 E6 and E7
]proteins, was tested for binding to 5 different HLA-A
molecules.

        The results of this analysis are depicted in Tables
20(a)-(d). Table 20(a) describes the peptides of HPV 16 that

20   bound to HLA-AL molecules. All peptides were tested. Listed
are only peptides yielding ratio values of ≥ 0.001. It can be
seen that 2 peptides bound with high affinity to this molecule
(>0.1), 6 with intermediate affinity (0.1-0.01) and 1 with low
affinity (0.01-0.001). Peptides were ranked by ratio value to

25   allow comparison of data obtained in different experiments.
To calculate the concentration of a peptide necessary to yield
a 50% inhibition dose (IC_{50}) one has to divide the value of
the standard IC_{50} by the ratio. For example, peptide E6-80
has an IC_{50} of 23 nM (81/3.5).

30   Table 20(b) describes the peptides that bound to HLA-
A3.2 molecules. Seven peptides were identified as high
affinity binders, 6 as intermediate affinity binders and 13 as
low affinity binders. Table 20(c) describes the peptides that
bound to HLA-A11.2 molecules. Six high affinity peptides were

35   identified, 4 intermediate affinity binders and 10 low
affinity binders. Two high affinity binding peptides (E6-59
IVYRDGNPY and E6-80 ISEYRHYAY) and two weak affinity binding
peptides with a Y at the 9th position (E6-42 QQLLRREVY, E6-69

66

VADKALKFY) were identified for HLA-A11.2 Considering the high
binding strength of the first two peptides and the similarity
between the HLA-A11.2 motif and the HLA-A3.2 motif in which
Y's are preferred at the 9th aa position, tyrosines should be
included at the 9th position in the HLA-A11.2 motif. Comparing
Tables 21(b) and (c) it is clear that there is a large overlap
of peptides that bound to both A3.2 and A11.2 molecules.
Eighteen out of 28 E6 and E7 peptides binding to these two HLA
molecules overlapped and only 8 peptides were unique for HLA-
A3.2 and 2 peptides unique for HLA-A11.2.

        Finally, Table 20(d) describes the peptides that
bound to HLA-A24 molecules. Here 2 peptides were identified
as high affinity binding peptides, 5 as intermediate affinity
binding peptides and 5 as low binding peptides. One high
affinity peptide (E6-72 KALKFYSKI) and one intermediate
affinity peptide (E7-49 RAHYNIVTF) were identified, indicating
that an A at the second position should be allowed in the HLA-
A24 motif. All these inclusions are indicated in Table 20-e.
In analyzing these tables it can be concluded that between 2
and 7 high affinity binding peptides were identified for all
of the tested HLA-A molecules. Occasionally some peptides
were binding to more alleles. Three peptides (E6-7, E6-37 and
E6-79), bound to HLA-A2.1, A3.2 and A11.2. One peptide (E6-
38) bound to HLA-A3.2, A11.2 and A24 and two peptides (E6-69
and E6-80) bound to HLA-A1, A3.2 and A11.2. But these
crossreactive peptides bound only weakly to one or more of the
different HLA molecules. In general, however, it can be
concluded that, except for HLA-A3.2 and HLA-A11.2 molecules,
almost all HLA molecules bind unique peptides.

        *Validation of HLA-A peptide binding motifs with an
unbiased set of HPV 16 E6 and E7 peptides.*

        We analyzed how well the motifs for anchor positions
described in this invention predicted the binding of a
peptide, and also the reverse: how well binding peptides
followed the identified motifs. For this, peptides were
ranked as high binders, intermediate binders, weak binders,
and negative binders and for each peptide the motif prediction
based on the anchor motif rules of Table 6 were analyzed. The

67

overall efficiency of the 2, (3), and 9 anchor motifs was then calculated and this is summarized in Table 20(e). It can be concluded that the motif described above for the different HLA-A molecules are quite accurate. One hundred percent of the HLA-A1, A3.2, and A24 high binders would be predicted as well as 67% of the HLA-11.2. Even for the intermediate binders between 40 and 100% would be predicted depending on the HLA-A molecule analyzed. Furthermore, the percent of weak binding peptides that would be predicted is low and the percent of those peptides that were predicted to bind but actually did not bind is very low for all these alleles.

Analyzed differently, of the 12 peptides predicted to bind to HLA-A1 actually 5 bound with high or intermediate affinity. This indicates that only a few peptides would have to be made to find these potential CTL epitopes. The figures for HLA-A3.2, A11.2, and A24 were 10/32, 7/26, and 4/7, respectively. This implies that the predictive value for all of these alleles is good. Besides a small number of peptides that had not been predicted by the recently described motifs, the (-) in Tables 21(a) - (d), a number of peptides that were predicted by the 2, (3), and 9 anchor motifs did not bind, indicating that having the right anchor residues is not always sufficient for binding and implicating that non-anchor residues can make negative contributions to the binding of a peptide.

Example 12
Presence of a Motif is Necessary But Not
Sufficient for High Affinity Class I Binding

To investigate further how the presence of different motifs might influence the capacity of different peptides to bind to the relevant HLA alleles, the sequences of various potential target molecules were scanned for the presence of motif-containing peptides. The peptides thus identified were synthesized and tested for binding. It was found (Table 20) that in the case of A3.2, only 39 (19%) of the 205 peptides bound with high affinity in the 1 to 50 nM range. 22.4% of them bound with intermediate affinities (in the 50 to 500 nM

68

range), while 34.6% bound weakly (in the 500 nM to 50 μM
range). Finally, 23.9% of them did not bind at all, at least
up to the 50 μM level. In the case of A11, 33 (33%) of the
100 peptides bound with high affinity in the 1 to 50 nM range.
5   35% of them bound with intermediate affinities (in the 50 nM
range.), while 24% bound weakly (in the 500 nM to 50 μM
range). Finally, 8% of them did not bind at all, at least up
to the 50 μM level.

Similar results were also obtained (data not shown)
10 in the case of A1 and A24.

The same type of analysis were also performed in the
case of 10-mer peptides carrying either the A3.2, and A11
motifs (Tables 22(a) and (b)). It was found that in these
cases, the frequency of good binders was even lower (17.5%,
15 and 29.8%, respectively). These data confirm the fact that
motif-containing 10-mer peptides can indeed bind, albeit with,
in general, reduced affinity.

In summary, the data shown in this section clearly
show that the presence of the correct anchor residues is not
20 sufficient per se to allow for good HLA binding. It is thus
apparent that the nature of the residues contained in
positions other than 2(3) and 9 (or 10) can influence binding.
The most likely explanation of this observation is that the
presence of certain residues (in positions other than 2 and 9)
25 can negate or increase the binding potential of a peptide
determinant.

The data shown in the preceding sections describe how
specific binding assays can be used to identify, within motif-
containing peptides, peptides that are immunogenic. We also
30 wanted to devise an alternative strategy, namely to derive
procedures that would be able to predict, within motif-
containing peptides, which peptides might be good or
intermediate binders and thereby might be immunogenic. In
other experiments not shown intermediate or good binders have
35 been shown to be immunogenic. In particular, to identify
residues that have a negative impact on binding an analysis of
all positions for A3.2, A11, and all motif-containing
peptides, both 9-mers and 10-mers is carried out. In the case

69

of A11, because of the small occurrence of nonbinding
peptides, a different cutoff was used such that the analysis
compares good and intermediate binders on the one hand to weak
and nonbinders on the other.

5

Example 13
Algorithms to Identify Immunogenic Peptides

In light of results presented in Example 13 above,
algorithms are developed to provide a more exact predictor of
10   binding based upon the effects of different residues at each
position of a peptide sequence, in addition to the anchor or
conserved residues. More specifically, we utilize the data
bank obtained during the screening of our collection of A1,
3,11 or 24 motif containing peptides to develop an algorithm
15   for each particular allele which assigns a score for each
amino acid at each position along a peptide. The score for
each residue is taken as the ratio of the frequency of that
residue in good and intermediate binders to the frequency of
occurrence of that residue in non-binders.

20   In the present algorithm residues have been grouped
by similarity. This avoids the problem encountered with some
rare residues, such as tryptophan, where there are too few
occurrences to obtain a statistically significant ratio. A
listing is made of scores obtained by grouping for each of the
25   twenty amino acids by position for 9-mer peptides containing
conserved residues that define their motif (2/9 motifs). A
peptide is scored in the algorithm as a product of the scores
of each of its residues.

The power of an algorithm to correlate with binding
30   is further underlined by its ability to predict a population
of peptides with the highest occurrence of good binders. If
one were to rely, for example, solely on the 2/9 motif for
predicting 9-mer peptides which bind to a specific MHC allele
the large number of peptides containing the motif would be
35   predicted to be good binders. In fact only a relatively small
percentage of these peptides are good binders and a somewhat
larger percentage are intermediate binders, while a still
larger percentage of the peptides predicted by the motif are

70

either weak or non-binding peptides. In contrast, using the grouped algorithm of this invention a population of peptides are created with a greater percentage of good binders, a still greater percentage of intermediate binders, and a smaller percentage, relative to that predicted by motif-containing peptides, are weak and non-binders.

The present example of an algorithm uses the ratio of the frequency of occurrence of an amino acid in binders and non-binders to measure the impact of a particular residue at each position of a peptide. It is immediately apparent to one of ordinary skill in the art that there are alternative ways of creating a similar algorithm. For example, one could use average binding affinity values, or relative binding of single amino acid substitutions in a motif containing peptide with a poly-alanine backbone to generate an algorithm table.

An algorithm using average binding affinity has the advantage of including all of the peptides in the analysis, and not just good/intermediate binders and non-binders. Moreover, it gives a more quantitative measure of affinity than the simpler group ratio algorithm. We create such an algorithm by calculating for each amino acid, by position, the average log of binding when that particular residue occurs in our set of motif containing peptides. The algorithm score for a peptide is then taken as the sum of the scores by position for each of its residues.

Example 14
Preparation of effective HLA allele-specific antigen presenting cells.

This example demonstrates the use of cold temperature incubation or acid stripping/peptide loading method to prepare effective HLA-allele-specific antigen presenting cells (APC). The APC were used to sensitize precursor cytotoxic T lymphocytes which led to the development of antigen-specific cytotoxic cells. This was accomplished using either phytohemaglutinin (PHA) T-cell blasts or peripheral blood mononuclear cells (PBMC) or staphylococcus aureus Cowan I (SAC-I) activated PBMC as APC. The results are applicable to other APC and to the other MHC alleles.

The following describes sources for materials used in the following examples:

L-Ascorbic acid, Cat #B582, J.T. Baker, Phillipsburg, NJ.

Anti-HLA A2 (BB7.2), Cat #HB82, ATCC, Rockville, MD.

Anti-HLA DR (LB3.1), from J. Gorga, Children's Hospital, Pittsburgh, PA.

Anti-HLA Alpha chain pan ABC (9.12.1), from R. DeMars, University of Wisconsin, Madison, Wl.

Anti-mouse IgG FITC conjugate, Cat #F2883, Sigma, St. Louis, MO.

$\beta_2$microglobulin, Cat #M0114, Scripps Labs, San Diego, CA.

BSA Fraction V, Cat #A9418, Sigma, St. Louis, MO.

50cc conical centrifuge tubes, Cat #2070, Falcon, Lincoln, Park, NJ.

Cryo 1°C freezing container, Cat #5100-0001, Nalge, Rochester, NY.

Cryovial, Cat #5000-0012, Nalge, Rochester, NY.

Dimethyl sulfoxide (DMSO), Cat #D2650, Sigma, St. Louis, MO.

DNase, Cat #260912, Calbiochem, San Diego, CA.

Dynabeads M-450 goat anti-mouse IgG, Cat #110.06, Dynal, Great Neck, NY.

EDTA tetrasodium salt, Cat #ED4SS, Sigma, St. Louis, MO.

FACScan, Becton Dickinson, San Jose, CA.

Fetal calf serum (FCS), Cat #3000, Irvine Scientific, Irvine, CA.

Ficoll-Paque, Cat #17-0840-03, Pharmacia, Piscataway, NJ.

Gentamicin, Cat #600-5750AD, Gibco, Grand Island, NY.

L-Glutamine, Cat #9317, Irvine Scientific, Irvine, CA.

GS-6KR centrifuge, Beckman Instruments, Palo Alto, CA.

Human AB serum (HS), Cat #100-112, Gemini Bioproducts, Calabasas, CA.

Human rIL-2, Sandoz, Basel, Switzerland.

Human rIL-7, Cat #F1-1587-1, Genzyme, Cambridge, MA.

Isopropanol, Cat #A464-4, Fisher Scientific, Pittsburgh, PA.

MicroCELLector T-150 culture flask for selection of CD4+ cells, Cat #8030, Applied Immune Sciences, Menlo Park, CA.

Micromedic automatic gamma counter, ICN Micromedics Systems, Huntsville, AL.

OKT4 hybridoma supernatant, Cat #CRL 8002, ATCC, Rockville, MD.

Paraformaldehyde, Cat #T-353, Fisher, Pittsburgh, PA.

PBS calcium and magnesium free (CMF), Cat #17-516B, BioWhittaker, Walkersville, MD.

Peptides used in this study were synthesized at Cytel and described in Table 24 a.

Phytohemagglutinin (PHA), Cat #HA-16, Wellcome, Dartford, England.

RPMI 1640 + Hepes + glutamine, Cat #12-115B, BioWhittaker, Walkersville, MD.

RPMI 1640 + Hepes + glutamine, Cat #380-2400AJ, Gibco, Grand Island, NY.

Sodium chloride (NaCl), Cat #3624-05, J.T. Baker, Phillipsburg, NJ.

Sodium (^{51}Cr) chromate, Cat #NEZ 030, NEN, Wilmington, DE.

Sodium phosphate monobasic, Cat #S9638, Sigma, St. Louis, MO.

Triton X-100, Cat #X-100, Sigma, St. Louis, MO.

24 well tissue culture plate, Cat #3047, Falcon, Becton Dickinson, San Jose, CA.

96 well U-bottomed cluster plate, Cat #3799, Costar, Cambridge, MA.

Culture Medium. PHA blasts and CTL inductions were done in RPMI 1640 + Hepes + glutamine (Gibco) supplemented with 2 mM L-glutamine (Irvine Scientific), 50 µg/ml gentamicin (Gibco), and 5% heat inactivated pooled human Type AB serum (Gemini

Bioproducts) [RPMI/5% HS]. EBV transformed lymphoblastoid
cell lines (LCL) were maintained in RPMI 1640 + Hepes +
glutamine (BioWhittaker) supplemented with L-glutamine and
gentamicin as above and 10% heat inactivated fetal calf serum
5   (Irvine Scientific) [RPMI/10% FCS]. Chromium release assays
were performed in RPMI/10% FCS.

Cytokines. Recombinant human interleukin-2 (rIL-2) (Sandoz)
was used at a final concentration of 10 U/ml. Recombinant
10 human interleukin-7 (rIL-7) (Genzyme) was used at a final
concentration of 10 ng/ml.

Isolation of Peripheral Blood Mononuclear Cells (PBMC). Whole
blood was collected in heparin (10 U/ml) containing syringes
15 and spun in 50cc conical centrifuge tubes (Falcon) at 1600 rpm
(Beckman GS-6KR) 15 min. The plasma layer was then removed
and 10 ml of the buffy coat collected with a 10 ml pipette
using a circular motion. The buffy coat was mixed thoroughly
and diluted with an equal volume of serum free RPMI 1640. The
20 diluted buffy coat was then layered over 20 ml Ficoll-Paque
(Pharmacia) in a 50cc conical tube and centrifuged 400 x g for
20 min at room temperature with the brake off. The
Ficoll-plasma interface containing the PBMCs was collected
using a transfer pipet (two interfaces per 50cc tube) and
25 washed three times with 50 ml RPMI (1700, 1500, and 1300 rpm
for 10 min.

Freezing and Thawing PBMC. PBMC were frozen at 30×10^6
cells/ml of 90% FCS + 10% DMSO (Sigma), in 1 ml aliquots using
30 cyrovials (Nalge). Cryovials were placed in Cryo 1°C freezing
containers (Nalge) containing isopropanol (Fisher) and placed
at -70°C from 4 hr (minimum) to overnight (maximum).
Isopropanol was changed after every 5 uses. Cryovials were
transferred to liquid nitrogen for long term storage. PBMC
35 were thawed by continuous shaking in a 37°C water bath until
the last crystal was nearly thawed. Cells were immediately
diluted into serum free RPMI medium containing DNAse 30 µg/ml
(to avoid clumping) (Calbiochem), and washed twice.

74

Depletion of Lymphocyte Subpopulations. CD4 lymphocyte
depletion was performed using antibody-coated flasks:
MicroCELLector T-150 flasks for the selection of CD4+ cells
(Applied Immune Sciences) were washed according to the
5   manufacturer's instructions with 25 ml PBS CMF + 1 mM EDTA
(Sigma) by swirling flasks for 30 sec followed by incubation
for 1 hr at room temperature on a flat surface. Buffer was
aspirated and flasks were washed 2 additional times by shaking
the flasks for 30 sec and maintaining coverage of the binding
10   surface. To each washed flask, 25 ml culture medium + 5% HS
were added and incubated for 20 min at room temperature on a
flat surface. Media was left in the flask until it was ready
to receive the cells. PBMC were thawed in RPMI/5% HS
containing 30 µg/ml DNAse, and washed twice. HS in the wash
15   blocks Fc receptors on PBMCS. For one flask a maximum of 12 x
10^7 cells were resuspended in 25 ml culture medium. Culture
medium was aspirated from the flask and then the cell
suspension was gently added to the MicroCELLector. Flasks
containing the cells were incubated for 1 hr at room
20   temperature on a flat surface. At the end of the incubation,
the flask was gently rocked from side to side for 10 sec to
resuspend the nonadherent cells. Nonadherent CD4 depleted
cells were harvested, and then flasks were washed twice with
PBS CMF to collect the nonadherent cells. Harvested
25   CD4-depleted cells were pelleted by centrifugation and
resuspended in complete culture medium (RPMI/5%/HS).

Generation of PHA Blasts. PBMC were isolated using the
standard Ficoll-Paque protocol. Frozen cells were washed
30   twice before use. Cells were cultured at 2 x 10^6/ml in
RPMI/5% HS containing 1 µg/ml PHA (Wellcome) and 10 U/ml
rIL-2. PHA blasts were maintained in culture medium
containing 10 U/ml r IL-2 with feeding and splitting as
needed. PHA blasts were used as APC on day 6 of culture.
35   Generation of empty class I molecules and peptide loading were
only performed by the acid strip method when using these APC.
Acid Stripping/Peptide Loading of PBMC and PHA Blasts. PBMC
were isolated using the Ficoll-Paque protocol. When using

frozen cells, PBMC were washed twice before using. PHA blasts
were prepared as previously described and washed twice before
using. Once cells were prepared, they were washed once in
cold sterile 0.9% NaCl (J.T. Baker) + 1% BSA. In a 50cc
conical centrifuge tube, the cells were resuspended at 10^7/ml
in cold sterile citrate-phosphate buffer [0-13 M L-ascorbic
acid (J.T. Baker), 0.06 M sodium phosphate monobasic (Sigma)
pH 3, 1% BSA, 3 µg/ml $\beta_2$microglobulin (Scripps Labs)] and
incubated for 2 min on ice. Immediately, 5 volumes of cold
sterile neutralizing buffer #1 [0.15 M sodium phosphate
monobasic pH 7.5, 1% BSA, 3 µg/ml $\beta_2$microglobulin, 10 µg/ml
peptide] were added, and the cells were pelleted at 1500 rpm,
5 min at 4°C. Cells were resuspended in 1 volume cold sterile
neutralizing buffer #2 [PBS CMF, 1% BSA, 30 lig/ml DNAse, 3
µg/ml $\beta_2$microglobulin, 40 µg/ml peptide] and incubated for 4
hrs at 20°C. Cells were diluted with culture medium to
approximately 5 x 10^6/ml and irradiated with 6000 rads. Cells
were then centrifuged at 1500 rpm for 5 min at room
temperature and resuspended in culture medium. The acid
stripped/peptide loaded cells were used immediately in the CTL
induction cultures (below).

Induction of Primary CTL using Acid Stripped/Peptide Loaded
Autologous PBMCs or PHA Blasts as Stimulators. Acid
stripping/peptide loading of PBMC and PHA blasts are described
above. During the last 4 hr incubation of stimulator cells
with peptide, the responder cell population was prepared:
Responders were PBMC that were depleted of CD4+ cells
(described above). Responder cells were resuspended in
culture medium at 3 x 10^6/ml. 1 ml of the responder cell
suspension was dispensed into each well of a 24-well tissue
culture plate (Falcon, Becton Dickinson). The plates were
placed in the incubator at 37°C, 5% CO_2 until the stimulator
population was ready. Once irradiated, stimulator APC were
resuspended in culture medium containing 20 ng/ml rIL-7 at
10^6/ml for the PBMC, or at 3 x 10^5/ml for the PHA blasts. 1
ml of stimulator cell suspension was added per well to the
plates containing the responders. On day 7 after induction, a

100 μl culture medium containing 200 ng/ml rIL-7 was added to each well (20 ng/well rIL-7 final). On day 10 after induction, 100 μl of culture medium containing 200 U/ml rIL-2 was added to each well (20 U/well rIL-2 final).

Antigen Restimulation of CTL. On day 12-14 after the induction, the primary CTL were restimulated with peptide using adherent APC. Autologous PBMC were thawed and washed as described above. Cells were irradiated at 6000 rads. Cells were pelleted and resuspended in culture medium at 4 x 10^6/ml. 1 ml of cell suspension was added to each well of a 24-well tissue culture plate, and incubated for 2 hrs at 37°C, 5% CO_2. Non-adherent cells were removed by washing each well three times with serum free RPMI. After this step, a 0.5 ml culture medium containing 3 μg/ml $\beta_2$microglobulin and 20 μg/ml total peptide was added to each well. APC were incubated for 2 hrs at 37°C, under 5% CO_2 with the peptide and $\beta_2$microglobulin. Wells were aspirated and 1 ml of responder cells at 1.5 x 10^6/ml in culture medium was added to each well. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added to each well.

Cytotoxicity Chromium Release Assay. Seven days following restimulation of primary induction, the cytotoxic activity of the cultures was assessed.
          a.   <u>Effector Cell Preparation</u>: the responders, which at this stage are renamed "effectors", were centrifuged and resuspended at 10^7/ml in RPMI/10% FCS. Three-fold serial dilutions of effectors were performed to yield effector to target ratios of 100:1, 33:1, 11:1, and 3:1. Effector cells were aliquoted at 100 μl/well on 96 well U-bottomed cluster plates (Costar), in duplicate.
          b.   <u>Target Cell Preparation</u>: Approximately 16-20 hrs prior to the assay, target cells were resuspended at 3 x 10^5/ml in RPMI/10% FCS in the presence or absence of 3 μg/ml $\beta_2$microglobulin and 10 μg/ml total peptide. After preincubation, target cells were centrifuged and pellets were resuspended in 200 μl (300μCi) sodium (^{51}Cr) chromate (NEN).

Cells were incubated at 37°C for 1 hr with agitation.
Labelled target cells were washed 3 times with RPMI/10% FCS.

       c. <u>Setting-Up the Assays</u>: Target cell concentration
was adjusted to 10^5/ml in RPMI/10% FCS and 100 µl aliquots
were added to each well containing responders. K562 cells
(cold targets, to block NK, and LAK activity) were washed and
resuspended in RPMI/10% FCS at 10^7/ml. Aliquots of 20 µl were
added per well, yielding a 20:1 of cold K562 target:labelled
target. For the determination of the spontaneous ^{51}Cr
release, 100 µl/well of RPMI/10% FCS were added to 100 µl/well
of labelled target cells, and 20 µl/well of K562. For maximum
^{51}Cr release, 100 µl 1% Triton X-100 (Sigma) in PBS CMF, was
added to the 100 µl/well labelled target cells, and 20 µl/well
K562. Plates were centrifuged for 2 min at 1200 rpm to
accelerate cell conjugate formation. Assays were incubated
for 5 hr at 37°C, 5% CO_2. Assays were harvested by
centrifuging plates for 5 min at 1200 rpm and collecting 100
µl/well of supernatant. Standard gamma counting techniques
were used to determine percent specific lysis (Micromedic
automatic gamma counter, 0.5 min per tube).

Cultured Cell Lines. JY, a HLA A2.1 expressing human
EBV-transformed B-cell line, was grown in RPMI/10% FCS. K562,
a NK cell sensitive erythroblastoma line was grown in RPMI/10%
FCS. K562 was used to reduce background killing by NK and LAK
cells in the chromium release assays.

Peptides. The peptides used in these studies were synthesized
at Cytel and their sequences are described in Table 24 a.
Peptides were routinely diluted in 100% DMSO at 20 mg/ml,
aliquoted, and stored at -20°C.

FACS Analysis. Approximately 10^6 cells were used for each
antibody that was to be tested. Cells were washed twice with
PBS CNU + 0.1% BSA. To each sample, 100 µl PBS CMF + 0.1% BSA
+ primary antibody at 2 µg/ml (BB7.2, ATCC) or (9.12.1,
Inserm-CNRS, Marseille, France) or (LB3.1, Children's Hospital
Pittsburgh) were added. A negative control was always

78

included. Cells were incubated on ice for 20 min and washed
twice with PBS CMF + 0.1% BSA. Cells were resuspended in 100
μl anti-mouse IgG FITC conjugate (Sigma), diluted 1:50 in PBS
CMF + 0.1% BSA, and incubated 20 min on ice. Cells were
5   washed twice with PBS CMF + 0.1% BSA, and resuspended in PBS
for FACScan (Becton Dickinson) analysis. When it was
necessary to postpone analysis to the subsequent days, the
cells were fixed with PBS/1% paraformaldehyde (Fisher) and
analyzed within one week.
10
Binding Assays Using Intact Cells and Radiolabelled Peptide.
JY cells were treated with citrate-phosphate buffer and
neutralizing buffer #1 as described above. JY control cells
were left untreated in tissue culture media. After treatment
15 both cell populations were washed twice with serum free RPMI
and loaded with ^{125}I-radiolabelled 941.01 (HBc15-27) peptide
(standard chloramine T iodination). To determine binding
specificity, 2 x 10^6 cells were resuspended in 200 μl
neutralizing buffer #2 (described above) containing
20 ^{125}I-941.01 (10^5 cpms) +/- 100 μg unlabelled 941.01. Cells
were incubated for 4 hrs at 20°C and washed twice with serum
free RPMI to remove free peptide. Cells were resuspended in
200 μl of serum free RPMI. In a microfuge tube the cell
suspension was layered over an 800 μl FCS and pelleted by
25 centrifugation for 5 sec. Supernatants were aspirated and the
radioactivity remaining in the pellet was measured (Micromedic
automatic gamma counter, 1 min per tube).

Example 15
30   Class I MHC molecule peptide stripping/loading by mild acid
treatment.

Mild acid solutions of pH 3 such as glycine or
citrate-phosphate buffers have been used by various groups to
identify endogenous peptides and to identify tumor associated
35 T cell epitopes. The treatment is unique in that while the MHC
class I molecules are destabilized (and peptides released),
while all other surface antigens remain intact including MHC
class II molecules. Most importantly, treatment of cells with

the mild acid solutions of this example do not affect the
cell's viability or metabolic state. The mild acid treatment
is rapid since the stripping of endogenous peptides occurs in
two minutes at 4°C and the APC is ready to perform its
function after the appropriate peptides are loaded. In this
example we utilized the technique to make peptide specific
APCs for the generation of primary antigen-specific CTL. The
resulting APC were efficient in inducing peptide-specific CD8+
CTL.

Measurements by FACS Analysis. PHA-induced T-cell blasts were
acid stripped/peptide loaded according to the methods
described in Example 15. The resulting cells were stained for
FACS analysis using anti-HLA-A2 (BB7.2) and anti-HLA alpha
chain-specific (9.12.1) monoclonal antibodies. Controls for
this experiment included the same cell population which was
not treated at pH 3 (but treated with PBS buffer at pH 7.2),
and with cells treated with citrate-phosphate buffer (to strip
the MHC) but neutralized in the absence of $\beta_2$microglobulin and
peptide. The results presented in Figure 15, indicate that
treatment of these cells with the citrate-phosphate (pH3)
buffer significantly reduced (10-fold) the reactivity of the
cells toward both anti-HLA class I antibodies alone
(anti-HLA-A2 and the alpha chain specific), but not towards a
monoclonal antibody specific for class II MHC molecules
(anti-HLA-DR). Most importantly, neutralization of the
acid-stripped cells in the presence of $\beta_2$microglobulin and
peptide resulted in preservation of a significant amount of
class I MHC antibody-reactive sites, with only a 2.5-fold
decrease in fluorescence intensity. Importantly, the
acid-treated cells remained viable, as measured by trypan blue
exclusion and forward/lateral FACS scatter analysis. Similar
results were obtained using EBV-transformed B cell lines,
fresh (or frozen) PBMC and other peptides (which bind to
either HLA-A2.1 or HLA-A1) (data not shown).

Binding of Radiolabeled Peptides to Empty MHC Molecules. To
determine the efficiency of peptide loading using the cold

temperature incubation or acid stripping/peptide loading
protocol, JY cells (an HLA-A2.1 EBV-transformed B cell line)
were preincubated at 26°C overnight or acid-stripped to remove
the endogenous MHC-associated peptides and the loading of
5   exogenous peptide was determined using a ^{125}I-radiolabelled
HLA-A2.1 binding peptide. The specificity of this reaction
was determined by measuring the inhibition of labelled peptide
binding using a cold peptide of the same sequence. Results
presented in Table 24 b demonstrate that acid-treatment of the
10   cells increased significantly (approximately 10-fold) the
amount of labelled peptide binding to the JY cells.
Furthermore, the binding of labelled peptide was completely
blocked by the addition of the cold peptide, demonstrating
specific binding (data not shown).
15

*In Vitro Induction of Primary Antigen-Specific CTL Using Acid
Stripped/Peptide Loaded APCS.* Additional critical parameters
for the induction of primary CTL using both the cold
temperature incubation and acid strip protocol are: 1)
20   enrichment of CD8+ T-cells in the responder cell population
(or depletion of CD4+ T-cells), 2) addition of rIL-7 to the
CTL induction cultures from day 0, and 3) restimulation of the
cultures with antigen on day 12-14 using autologous adherent
cells pulsed with peptide.   Results presented in Figs. 16 and
25   17 show experiments performed using PBMC and PHA-induced T-
cell blasts as APC. Figure 18 shows experiments using PHA-
induced T-cell blasts as APC while Figure 19 shows the use of
PBMC as APC.

30   Example 16
             Screening peptides to identify CTL epitopes.
             In order to identify CTL epitopes, CTL was stimulated
by SAC-I activated PBMCs as APC. Cold temperature expression
of the MHC in which class 1 β-2-microglobulin complex is
35   unstable was utilized in addition to acid stripping to
generate PBMC APC.

81

Complete Culture Medium. The tissue culture medium used in
this study consisted of RPMI 1640 with Hepes and L-glutamine
(Gibco) supplemented with 2 mM L-glutamine (Irvine
Scientific), 0.5mM sodium pyruvate (Gibco), 100 U/100 ug/ml
5   penicillin/streptomycin (Irvine), and 5% heat-inactivated
Human Serum Type AB (RPMI/5% HS; Gemini Bioproducts). Culture
media used in the growth of EBV-transformed lines contained
10% heat-inactivated fetal calf serum (RPMI/10% FCS, Irvine)
instead of human serum.

10
Cytokines. Recombinant human Interleukin-2 (rIL-2) and
Interleukin-4 (rIL-4) were obtained from Sandoz and used at a
final concentration of 10 U/ml and 10 ng/ml, respectively.
Human interferon-γ (IFN-γ) and recombinant human Interleukin-7
15   (rIL-7) were obtained from Genzyme and used at 20 U/ml and 10
ng/ml, respectively.

Peptides. Peptides were synthesized at Cytel and are
described in Table 24 a. Peptides were routinely diluted in
20   100% DMSO at 20 mg/ml, aliquoted, and stored at -70°C until
use.

Cell Lines. JY, Steinlin, EHM, BVR, and KT3 are homozygous
human EBV-transformed B cell lines expressing HLA $A_{2.1}$, A_1, A_3,
25   A_{11}, and A_{24}, respectively. They are grown in RPMI/10% FCS.
K562, an NK cell sensitive, erythoblastoma line grown in
RPMI/10% FCS, was used for reduction of background killing in
CTL assays. Melanoma cell lines either expressing the MAGE
antigen, mel 397 and mel 938, or not expressing the MAGE
30   antigen; mel 888, were also grown in RPMI/10% FCS.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs).
Whole blood was collected into heparin containing syringes and
spun in 50cc tubes at 1600 RPM (Beckman GS-6KR) for 15
35   minutes. The plasma layer was then removed and 10 ml of buffy
coat was collected with a pipette using a circular motion.
The buffy coat was mixed well and diluted with an equal volume
of RPMI. The buffy coat (30 ml) was then layered on 20 ml of

Ficoll-Paque (Pharmacia) and centrifuged at 1850 RPM (400xg)
for 20 minutes, 25°C, with the brake off. The interface
between the Ficoll and the plasma containing the PBMCs was
recovered with a transfer pipet (two interfaces per 50 ml
5   tube) and washed three times with 50 ml of RPMI (1700, 1500,
and 1300 RPM for 10 minutes). Cells were resuspended in 10-20
ml of culture medium, counted, and adjusted to the appropriate
concentration.

10 *Freezing PBMCs.* 30 million cells/tube (90% FCS/10% DMSO;
Sigma) were inserted into a Nalgene Cryo 1°C Freezing
Container containing isopropanol (Fisher) and placed at -70°C
from 4 hrs (minimum) to overnight (maximum). The isopropanol
was changed every five times. Tubes were transferred to
15 liquid nitrogen for long term storage. To thaw, PBMCs were
continuously shaken in a 37°C water bath until the last
crystal was almost thawed (tubes were not allowed to sit in
the water bath or at room temperature for any period of time).
Cells were diluted into serum-free RPMI containing 30 μg/ml
20 DNase to prevent clumping by dead cell DNA and washed twice.

Induction of Primary CTL Using SAC-I Activated PBMCs as APCs
    a. <u>Preparation of APCs</u>: PBMCs were purified using the
standard Ficoll-Paque protocol and resuspended at 1 x 10^6/ml
25 in RPMI/5% FCS containing 0.005% Pansorbin cells (SAC-I cells
expressing Protein A; Calbiochem), 20 μg/ml Immunobeads
(Rabbit anti-Human IgM; Biorad), and 20 ng/ml of human rIL-4.
Two ml of cells per well were plated in a 24-well plate
(Falcon, Becton Dickinson) and cultured at 37°C. After 3
30 days, the medium was removed and the cells were washed three
times followed by addition of RPMI/10% HS. The cells were
used after culturing for an additional 2 days in RPMI/10% HS.

    b. <u>Expression of empty Class I molecules on the surface of</u>
35 <u>APCs and peptide loading of APCs.</u>
        1. Cold temperature incubation:
            a. Expression of empty MHC in APCs: The APCs

were adjusted to a concentration of 2×10^6/ml in complete
culture medium containing 10 ng/ml rIL-4, 20 U/ml human IFN-γ,
and 3 μg/ml β2-microglobulin (β_2m; Scripps Lab). The cells
were then incubated overnight at 26°C in the presence of 5%
CO$_2$. It should be noted that these cells only express a
fraction of Class I molecules in the empty state ($~10\%$).

              b.   Peptide loading of APC stimulator cells:
  Empty Class I expressing APCs were washed 1-2 times with
serum free RPMI (+ L-glutamine and Hepes) and resuspended at 1
$\times 10^7$ in serum-free RPMI containing 50 μg/ml total of the
peptide pool (i.e., 16.7 μg/ml of each peptide in a pool of
three; 25 μg/ml of each peptide in a pool of two; 50 μg/ml of
individual peptide), 30 μg/ml DNAse, and 3 μg/ml β_2m.
Following a 4 hour incubation at 20°C, the cells were
irradiated at 6100 rads (5 $\times 10^6$/ ml; 25 million cells/tube),
washed and adjusted to the appropriate concentration for
addition to the induction culture (see below).

          2.   Acid stripping: This was used as an
  alternative method for generating empty MHC on the surface of
the APCs. The SAC-I activated PBMCs were washed once in cold
0.9% sodium chloride (J.T. Baker) containing 1% BSA. The
cells were resuspended at 10^7/ml in cold citrate-phosphate
buffer (0.13M L-ascorbic acid [J.T. Baker], 0.06M sodium
phosphate monobasic [Sigma], pH3) containing 1% BSA and 3
μg/ml β_2m and incubated on ice. After 2 minutes, 5 volumes of
cold 0.15M sodium phosphate monobasic buffer, pH7.5,
containing 1% BSA, 3 μg/ml β_2m, and 10 μg/ml peptide
[neutralizing buffer #1] was added and the cells centrifuged
at 1500 RPM for 5 minutes at 4°C. The cells were resuspended
in 1 ml of cold PBS containing 1% BSA, 30 μg/ml DNAse, 3 μg/ml
$\beta_2$microglobulin, and 50 μg/ml peptide [neutralizing buffer #2]
and incubated for 4 hours at 20°C. As above, subsequent to
the four hour incubation at 20°C, the cells were irradiated at
6100 rads (5 $\times 10^6$/ ml; 25 million cells/tube), washed, then
adjusted to the appropriate concentration for addition to the
induction culture (see below).

84

c.   Preparation of the CD4+ depleted PBMC responder cell
population (depletion of lymphocyte sub-populations using
AIS flasks). AIS MicroCellector T-150 flasks (specific
for the depletion of CD4+ T cells; Menlo Park, CA) were
primed by adding 25 ml of PBS/1 mM EDTA, swirling for 30
seconds so that all surfaces were moistened, and then
incubating with the binding surface down at room temperature
for 1 hour. Following this incubation, flasks were shaken
vigorously for 30 seconds, washed 1 time with PBS/EDTA, 2
additional times with PBS and then incubated with 25 ml of
culture medium for 15 minutes. PBMCs were thawed in
serum-free RPMI (+ L-glutamine + Hepes) containing 30 µg/ml
DNAse, washed once, and incubated for 15 minutes in culture
medium. Following aspiration of culture medium from the
flasks, up to 180 million PBMCs were added in 25 ml of culture
medium containing 30 µg/ml DNAse. After 1 hour at room
temperature, the flasks were rocked gently for 10 seconds to
resuspend the nonadherent cells. The nonadherent cell
suspension containing the CD8+ T cells was collected and the
flasks were washed 2 times with PBS. The CD4+ T cell depleted
PBMCs were centrifuged and counted for addition to the
induction culture. The CD4+ and CD8+ phenotype of the CD4+
depleted cell population was determined by FACS analysis (see
below). In general, this technique resulted in a two-fold
enrichment for CD8+ T cells with an average of approximately
40-50% CD8+ T cells and 15-20% remaining CD4+ T cells
following depletion of CD4+ T cells. Depletion of CD4+ T
cells can also be accomplished by antibody and complement or
antibody coated magnetic beads (Dynabeads). Depletion of CD4+
T cells served the purpose of enriching CTLp and removing
cells which would complete for cell nutrients and may
interfere with CTLp expansion.

d.   Induction of primary CTL. During the 4 hour peptide
loading of the stimulator APCs, CD4+ depleted PBMC to be used
as the responder population were prepared utilizing AIS flasks
for selection of CD8+ T cells through the depletion of CD4+ T
cells (above). The responder cells were plated at 3 x 10^6/ml
in a 1 ml volume (24 well plate) and placed at 37°C until the

peptide loaded stimulator APCs were prepared. The irradiated,
peptide loaded APCs were washed 1 time in serum-free RPMI (+
L-glutamine and Hepes), adjusted to 1×10^6/ml in complete
medium, and plated into a 24 well plate at 1 ml/plate: For

5   PBMC, 1×10^6 stimulator cells (1 ml volume) were plated into
the wells containing the responder cells; For SAC-I activated
PBMC and PHA blasts, 1 ml of 3×10^5/ml stimulator cells were
plated in each well. A final concentration of 10 μg/ml of
additional peptide was added in addition to 10 ng/ml final

10 concentration of rIL-7 (2 ml total volume). On day 7 an
additional 10 μg/ml rIL-7 was added to the culture and 10 U/ml
rIL-2 was added every 3 days thereafter. On day 12, the
cultures were restimulated with peptide pulsed adherent cells
and tested for cytolytic activity 7 days later (below).

15

Protocol for Restimulation of Primary CTL Using Adherent APC.
PBMCs were thawed into serum-free RPMI (+ L-glutamine and
Hepes) containing 30μg/ml DNAse, washed 2 times, and adjusted
to 5×10^6 /ml in culture medium containing DNAse. PBMCs (25

20 million cells/tube in 5 ml) were irradiated at 6100R. After 1
wash, the PBMCs were resuspended in culture medium and
adjusted to 4×10^6/ml. 1 ml of irradiated PBMCs was added
per well of a 24-well plate. The PBMC were incubated for 2
hours at 37°c, washed 3 times to remove non-adherent cells,

25 and cultured in medium containing 20 μg/ml total peptide and 3
μg/ml $\beta_2$microglobulin added in a 0.5 ml volume and again
incubated for 2 hours at 37°C. The peptide was aspirated and
1.5×10^6 responder cells resuspended in culture medium were
added in a 1 ml volume. After 2 days, 1 ml of culture medium

30 containing 20 U/ml rIL-2 was added.

FACS Analysis. One million cells/tube were centrifuged,
resuspended in 100 ul/tube PBS/0.1% BSA/0.02% sodium azide
(Sigma) plus 10 ul/tube directly conjugated antibody (Becton

35 Dickinson), and incubated on ice 15-20 minutes. Cells were
then washed 2 times with PBS/0.1% BSA/0.02% sodium azide and
resuspended in PBS to analyze on FACScan (Beckton Dickinson).
When it was not possible to analyze samples within 1-2 days,

cells were fixed with PBS containing 1% paraformaldehyde
(Fisher) and analyzed within one week.

Cytotoxicity Assay

  a. <u>Target cell preparation</u>. Approximately 16-20 hours
prior to the CTL assay, target cells (Class I matched
EBV-transformed lines) were washed once and resuspended in a
10 ml volume at 3×10^5/ml in RPMI/5% FCS in the presence or
absence of 10 µg/ml total peptide.

  b. <u>Labeling of target cells</u>: Target cells were
centrifuged and resuspended in 200 µl/tube sodium ^{51}Cr
chromate (NEN), then incubated at 37°C for 1 hour on a shaker.
Targets were washed 3 times (10 ml/wash) with RPMI/10% FCS and
resuspended in 10 ml (to determine the efficiency of
labelling, 50 µl/target was counted on the Micromedic
automatic gamma counter).

  c. <u>CTL assay</u>. Target cells were adjusted to 2×10^5/ml
and 50 µl of the cell culture was added to each well of a
U-bottomed 96-well plate (Costar Corp.) for a final
concentration of 1×10^4/well. K562 cells were washed once,
resuspended at 4×10^6/ml, and 50 µl/well was added for a
final concentration of 2×10^5/well (ratio of cold K562 to
target was 20:1). Responder cells were washed once,
resuspended at 9×10^6/ml, and three fold serial dilutions
were performed for effector to target ratios of 90:1, 30:1,
10:1, and 3:1. Responder cells were added in a volume of 100
µl in duplicate wells. For spontaneous release, 50 µl/well of
labelled target cells, 50 µl/well K562, and 100 µl/well of
medium was added. For maximum release, 50 µl/well target, 50
µl/well K562, and 100 µl/well of 0.1% Triton-X100 (Sigma) was
added. Plates were centrifuged for 5 minutes at 1200 RPM.
Following a 5 hour incubation at 37°C, plates were centrifuged
again for 5 minutes at 1200 RPM, and 100 µl/well of
supernatant was collected. Standard gamma counting techniques
(Micromedic automatic gamma counter; 0.5 minutes/tube) were
used to determine the percent specific lysis according to the
formula: % specific lysis = cpm experimental - cpm spontaneous
release/cpm maximum release - cpm spontaneous release X 100.

A cytotoxicity assay (CTL assay) was considered positive if
the lysis by CTL of targets sensitized with a specific peptide
at the two highest effector to target (E:T) ratios was 15%
greater than lysis of control targets (i.e., target cells

5   without peptide). A cytotoxicity assay (CTL assay) was
considered borderline if the lysis by CTL of targets
sensitized with a specific peptide at the two highest effector
to target (E:T ratios was 6% greater than lysis of control
targets (i.e., target cells without peptide).

10   d.   Results. Of the peptides that bind to the indicated
alleles, 9 of the 49 MAGE peptides, 10 of the 45 HIV peptides,
3 of the 25 HCV peptides, and 2 of the 20 HBV peptides tested
to date induced primary CTL in vitro. Representative graphs
illustrating CTL responses to various immunogenic peptides are

15   shown for MAGE (Figure 22), HIV (Figure 23), HCV (Figure 24),
and HBV (Figure 2). The CTL induction data are summarized in
Table 24 which lists the immunogenic peptides which bind to
the appropriate MHC and induce primary CTL in vitro.
Indicated is the peptide's sequence, corresponding antigen and

20   HLA allele to which it binds. Results shown in Figure 20
illustrate lysis of peptide sensitized targets following
stiumlation with SAC-I activated PBMCs
loaded with a MAGE 3 peptide, 1044.07 by the cold temperature
and incubation technique. Figure 21 shows a comparison of the

25   acid strip loading technique (Panel a) with the cold
temperature incubation technique (panel b).

          Although the present invention has been described in
some detail by way of illustration and example for purposes of

30   clarity and understanding, it will be apparent that certain
changes and modifications may be practiced within the scope of
the appended claims.

TABLE 13

VALIDATION OF CYTEL'S HLA MOTIFS

Binding Capacity (IC_{50}nM)						
Sequence	Motif	A1	A2.1	A3.2	A11	A24
AADKAAAAY	A1	50	--*	--	--	--
ATAKAAAAY	A1	15	--	329	77	--
ATDKAAAAY	A1	2.8	--	9250	840	ND
ALAKAAAAV	A2.1	--	125	--	--	--
AMAAAAAAK	A3.2	--	--	48	8.4	--
ATAAAAAAK	A11	--	--	59	40	--
AYAKAAAAF	A24	--	--	--	--	115

*A dash indicates an IC_{50} greater than 20,000 nM.

TABLE 14

VALIDATION OF CYTEL'S HLA MOTIFS

Binding Capacity (IC_{50} nM)					
SEQUENCE	MOTIF	A1	A2.1	A3.2	A11
AADKAAAAAY	A1	45	--*	--	--
ATAKAAAAAY	A1	58	--	1100	1030
ATDKAAAAAY	A1	4.0	--	10000	4533
ALAKAAAAAV	A2.1	ND	1400	--	--
AMAAAAAAAK	A3.2	ND	--	85	24.0
ATAAAAAAAK	A11	--	--	216	88

*A dash indicates an IC_{50} greater than 20,000 nM.

TABLE 15

HLA-A3.2

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 952.25	SUBSTITUTION
952.25	ALAAAAAAK	1	-
952.26	AMAAAAAAK	1.2	position 2
952.23	AVAAAAAAK	0.95	
981.04	ASAAAAAAK	0.89	
952.24	AIAAAAAAK	0.57	
952.27	AAAAAAAAK	0.57	
981.06	ATAAAAAAK	0.49	
981.08	AFAAAAAAK	0.13	
981.09	AGAAAAAAK	0.077	
981.13	ACAAAAAAK	0.031	
981.12	ADAAAAAAK	0.014	
981.11	ANAAAAAAK	0.0010	
981.05	AKAAAAAAK	<0.0016	
981.07	AYAAAAAAK	<0.0005	
981.10	APAAAAAAK	<0.0006	
952.35	ALAAAAAAR	0.46	position 9
981.36	ALAAAAAAY	0.15	
981.33	ALAAAAAAA	0.0034	
981.35	ALAAAAAAQ	<0.0006	
981.37	ALAAAAAAS	<0.0005	
981.38	ALAAAAAAT	<0.0005	
981.34	ALAAAAAAN	<0.0005	
981.39	ALAAAAAAE	<0.0003	

90

TABLE 16

HLA-A11

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 952.25	SUBSTITUTION
952.25	ALAAAAAAK	1	-
952.26	AMAAAAAAK	2.5	position 2
952.27	AAAAAAAAK	1.1	
952.24	AIAAAAAAK	0.72	
981.06	ATAAAAAAK	0.55	
981.04	ASAAAAAAK	0.46	
981.09	AGAAAAAAK	0.38	
952.23	AVAAAAAAK	0.23	
981.11	ANAAAAAAK	0.23	
981.13	ACAAAAAAK	0.019	
981.08	AFAAAAAAK	0.020	
981.12	ADAAAAAAK	0.012	
981.05	AKAAAAAAK	0.0065	
981.07	AYAAAAAAK	<0.0065	
981.10	APAAAAAAK	<0.0051	
952.35	ALAAAAAAR	0.015	position 9
981.33	ALAAAAAAA	<0.0059	
981.34	ALAAAAAAN	<0.0071	
981.35	ALAAAAAAQ	<0.0051	
981.36	ALAAAAAAY	<0.0071	
981.37	ALAAAAAAS	<0.0051	
981.38	ALAAAAAAT	<0.0051	
981.39	ALAAAAAAE	<0.0071	

9 1

TABLE 17

HLA-A24

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 983.01	SUBSTITUTION
983.01	AYAKAAAAF	1	-
983.08	AFAKAAAAF	0.24	position 2
983.09	APAKAAAAF	0.0058	
983.10	AAAKAAAAF	0.0023	
983.11	AKAKAAAAF	<0.0012	
983.05	AYAKAAAAI	0.20	position 9
983.04	AYAKAAAAL	0.11	
983.06	AYAKAAAAV	0.0023	
983.02	AYAKAAAAA	<0.0012	
983.03	AYAKAAAAY	<0.0012	
983.07	AYAKAAAAK	<0.0012	

TABLE 18

HLA-A1

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 982.07	SUBSTITUTION
982.011	ATDKAAAAY	Motif	-
982.07	ATAKAAAAY	1	-
982.09	ASAKAAAAY	0.17	position 2
982.13	AMAKAAAAY	0.095	no D in pos 3
982.08	AAAKAAAAY	0.0064	
954.09	ALAKAAAAY	0.0045	
954.11	AIAKAAAAY	0.0045	
954.13	AVAKAAAAY	0.0020	
982.10	AKAKAAAAY	0.0011	
982.11	ANAKAAAAY	<0.0001	
982.12	ADAKAAAAY	<0.0001	
982.14	AGAKAAAAY	<0.0001	
982.15	APAKAAAAY	<0.0001	
982.16	AYAKAAAAY	<0.0001	
982.17	AHAKAAAAY	<0.0001	
982.24	ATAKAAAAA	0.0040	position 9
982.23	ATAKAAAAF	0.0019	no D in pos 3
982.28	ATAKAAAAH	0.0010	
982.32	ATAKAAAAV	0.0005	
982.25	ATAKAAAAN	<0.0001	
982.26	ATAKAAAAD	<0.0001	
982.27	ATAKAAAAW	<0.0001	
982.30	ATAKAAAAK	<0.0001	
982.31	ATAKAAAAI	<0.0001	
982.29	ATAKAAAAP	<0.0001	

TABLE 19

HLA-A1

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 982.07	SUBSTITUTION
982.01	ATDKAAAAY	Motif	-
982.07	ATAKAAAAY	1	-
982.01	AADKAAAAY	0.14	position 3
954.03	AAEKAAAAY	0.038	no T in pos 2
982.02	AAAKAAAAY	0.0055	
982.06	AASKAAAAY	0.0024	
982.04	AANKAAAAY	0.0011	
982.03	AAQKAAAAY	0.0008	
982.05	AAKKAAAAY	<0.0001	
982.20	AADKAAAAA	0.0016	position 9
982.21	AADKAAAAW	0.0005	no T in pos 2
982.19	AADKAAAAF	<0.0001	
982.22	AADKAAAAK	<0.0001	

94

TABLE 20(A)

HPV16 E6 AND E7 PEPTIDES BINDING TO HLA-A1

Orgin	First aa Position	Sequence*	Binding Ratio to Standard‡	Motif Prediction
E6	80	ISEYRHYAY	3.500	+
E6	69	VADKALKFY	0.240	+
E7	44	QAEPDRAHY	0.029	+
E7	37	EIDGPAGQA	0.025	-
E7	19	TTDLYAYEQ	0.023	+
E6	144	MSAARSSRT	0.019	+/-
E7	73	HVDIRTLED	0.014	-
E6	139	WTGRAMSAA	0.010	-
E6	61	YRDGNPYAV	0.008	-

* Bold A's indicate residues in which cysteine was replaced by alanine.

‡ The average IC$_{50}$ value ±SE of the standard in the course of the experiments considered in this table was 81±30 nM. Listed in the table are peptides yielding ratio values of ≥0.001. All other peptides yielded ratio values of ≤0.001.

TABLE 20(B)

HPV16 E6 and E7 Peptides Binding to HLA-A3.2

Origin	First aa Position	Sequence*	Binding Ratio to Standard‡	Motif Prediction
E6	107	LIRAINAQK	3.7000	+
E6	59	IVYRDGNPY	3.0000	+
E7	89	IVAPIASQK	2.2000	+
E6	33	IILEAVYAK	1.5000	+
E6	125	HLDKKQRFH	0.4400	+
E6	143	AMSAARSSR	0.1800	+
E6	7	AMFQDPQER	0.1000	+
E6	93	TTLEQQYNK	0.0780	+
E6	37	AVYAKQQLL	0.0320	-
E7	51	HYNIVTFAA	0.0210	-
E6	145	SAARSSRTR	0.0200	+
E6	75	KFYSKISEY	0.0100	+
E6	89	SLYGTTLEQ	0.0080	-
E7	52	YNIVTFAAK	0.0067	-
E6	80	ISEYRHYAY	0.0064	+
E6	42	QQLLRREVY	0.0058	-
E6	68	AVADKALKF	0.0056	+
E6	97	QQYNKPLAD	0.0045	-
E6	79	KISEYRHYA	0.0044	-
E6	84	RHYAYSLYG	0.0036	-
E6	69	VADKALKFY	0.0025	+
E6	146	AARSSRTRR	0.0020	+
E7	58	AAKADSTLR	0.0016	+
E6	38	VYAKQQLLR	0.0012	-
E6	67	YAVADKALK	0.0012	+
E7	60	KADSTLRLA	0.0012	-

* Bold A's indicate residues in which cysteine was replaced by alanine.

‡ The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 30±3 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of ≤0.001.

Table 20(C)

HPV16 E6 and E7 Peptides Binding to HLA-A11.2

Orgin	First aa Position	Sequence*	Binding Ratio to Standard‡	Motif Prediction
E6	33	IILEAVYAK	6.7000	+
E6	93	TTLEQQYNK	1.8000	+
E7	89	IVAPIASQK	1.3000	+
E6	7	AMFQDPQER	0.8400	+/-
E6	59	IVYRDGNPY	0.4700	- (+)§
E6	80	ISEYRHYAY	0.4300	- (+)§
E6	37	AVYAKQQLL	0.0450	-
E6	145	SAARSSRTR	0.0330	+/-
E6	107	LIRAINAQK	0.0120	+
E7	58	AAKADSTLR	0.0110	+/-
E6	42	QQLLRREVY	0.0084	+/- (+)§
E6	143	AMSAARSSR	0.0084	-
E6	79	KISEYRHYA	0.0076	-
E6	67	YAVADKALK	0.0074	+
E7	52	YNIVTFAAK	0.0060	+
E6	68	AVADKALKF	0.0037	-
E6	69	VADKALKFY	0.0030	- (+)§
E6	38	VYAKQQLLR	0.0022	+/-
E6	140	TGRAMSAAR	0.0012	+/-
E7	90	VAPIASQKP	0.0012	-
E7	51	HYNIVTFAA	0.0010	-

* Bold A's indicate residues in which cysteine was replaced by alanine.

‡ The average IC$_{50}$ value ±SE of the standard in the course of the experiments considered in this table was 10±3 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of ≤0.001.

§ Brackets indicate score according to adjusted motif.

Table 20(D)

HPV16 E6 and E7 Peptides Binding to HLA-A24

Orgin	First aa Position	Sequence*	Binding Ratio to Standard‡	Motif Prediction
E6	87	AYSLYGTTL	0.1200	+
E6	72	KALKFYSKI	0.1100	- (+)§
E6	131	RFHNIRGRW	0.1000	+
E7	49	RAHYNIVTF	0.0670	- (+)§
E6	49	VYDFAFRDL	0.0610	+
E6	82	EYRHYAYSL	0.0460	+
E6	26	LQTTIHDII	0.0200	-
E6	66	PYAVADKAL	0.0055	-
E6	1	MHQKRTAMF	0.0049	-
E6	85	HYAYSLYGT	0.0037	-
E6	44	LLRREVYDF	0.0023	+
E6	38	VYAKQQLLR	0.0011	-

* Bold A's indicate residues in which cysteine was replaced by alanine.
‡ The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 22±6 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of ≤0.001.
§ Brackets indicate score according to adjusted motif.

TABLE 20(B)

SUMMARY OF EFFICACY OF 2, (3), 9 ANCHOR MOTIFS

Percentage Binders with Motifs	HLA Allele							
	A1		A3.2		A11.2		A24	
	Predicted/Found		Predicted/Found		Predicted/Found		Predicted/Found	
High (≥ 0.1)	2	2(100%)	7	7(100%)	6	6(100%)	2	3(67%)
Intermediate (0.1-0.01)	3	6(50%)	3	5(60%)	3	4(75%)	2	4(50%)
Weak (0.01-0.001)	0	1(0%)	6	14(43%)	6	11(55%)	2	5(20%)
Negative (≤ 0.001)	7	231(3%)	16	214(7%)	14	219(6%)	1	228(1%)
Totals	12	240	32	240	26	240	7	240

TABLE 21(A)

A3.2 9-MER OPTIMAL MOTIFS

9-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
IK	3(15%)	4(20%)	7(35%)	6(30%)	20(100%)
IR	3(15.8%)	4(21.1%)	7(36.8%)	5(26.3%)	19(100%)
LK	13(48.1%)	6(22.2%)	5(18.5%)	3(11.1%)	27(100%)
LR	4(8.5%)	9(19.1%)	20(42.6%)	14(29.8%)	47(100%)
SK	0	0	0	0	0
SR	0	0	0	1(100%)	1(100%)
TK	3(15%)	6(30%)	7(35%)	4(20%)	20(100%)
TR	2(16.7%)	1(8.3%)	2(16.7%)	7(58.3%)	12(100%)
VK	9(28.1%)	13(40.6%)	9(28.1%)	1(3.1%)	32(100%)
VR	2(7.4%)	3(11.1%)	14(51.9%)	8(29.6%)	27(100%)
Total	39(19%)	46(22.4%)	71(34.6%)	49(23.9%)	205(100%)

TABLE 21(B)
All 9-MER OPTIMAL MOTIFS

9-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
GK	0	1(100%)	0	0	1(100%)
IK	5(25%)	5(25%)	7(35%)	3(15%)	20(100%)
LK	6(22.2%)	10(37%)	9(33.3%)	2(7.4%)	27(100%)
TK	10(50%)	4(20%)	4(20%)	2(10%)	20(100%)
VK	12(37.5%)	15(46.9%)	4(12.5%)	1(3.1%)	32(100%)
Total	33(33%)	35(35%)	24(24%)	8(8%)	100(100%)

TABLE 22(A)

A3.2 10-MER OPTIMAL MOTIFS

10-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
AK	1(33.3%)	1(33.3%)	1(33.3%)	0	3(100%)
AR	0	0	1(100%)	0	1(100%)
FK	0	0	1(25%)	3(75%)	4(100%)
FR	0	6(27.3%)	10(45.5%)	6(27.3%)	22(100%)
IK	1(7.1%)	1(7.1%)	2(14.2%)	10(71.4%)	14(100%)
IR	16(53.3%)	7(23.3%)	5(16.7%)	2(6.7%)	30(100%)
LK	4(12.5%)	9(28.1%)	11(34.3%)	8(25%)	32(100%)
LR	1(100%)	0	0	0	1(100%)
MK	1(100%)	0	0	0	1(100%)
MR	2(11.8%)	0	8(47.1%)	2(11.8%)	17(100%)
TK	1(4.8%)	5(29.4%)	9(42.9%)	10(47.6%)	21(100%)
TR	7(35%)	1(4.8%)	5(25%)	4(20%)	20(100%)
VK	0	4(20%)	5(25%)	7(25%)	20(100%)
VR		6(21.4%)	15(53.6%)	7(25%)	28(100%)
Total	14(17.5%)	40(20.6%)	68(35.1%)	52(26.8%)	194(100%)

TABLE 22(B)

All 10-MER OPTIMAL MOTIFS

10-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
AK	1(33.3%)	1(33.3%)	1(33.3%)	0	3(100%)
CK	0	0	1(100%)	0	1(100%)
GK	0	0	0	0	0
IK	4(18.2%)	5(22.7%)	12(54.5%)	1(4.5%)	22(100%)
LK	9(30%)	12(40%)	8(26.7%)	1(3.3%)	30(100%)
MK	1(100%)	0	0	0	1(100%)
TK	6(35.3%)	5(29.4%)	5(29.4%)	1(5.9%)	17(100%)
VK	7(35%)	8(40%)	4(20%)	1(5%)	20(100%)
Total	28(29.8%)	31(33%)	31(33%)	4(4.3%)	94(100%)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A2.4
1.0300	HLDMLRHLY	9	c-ERB2			42	1	9.1	0.057	0.0002	
1.0346	LLDIOETEY	9	c-ERB2			849	1	7.6	0.0003	0	
1.0305	CTQLFEDNY	9	c-ERB2			104	1	0.18	0	0.028	
1.0055	LTCSPQFEY	9	c-ERB2			1131	1	0.13	0	0.0061	
1.0317	ETLEEITGY	9	c-ERB2			401	1	0.063			
1.0749	FTHQSDVWSY	10	c-ERB2			899	1	2.7	0.0003	0.0005	
1.0747	RLLDIDETEY	10	c-ERB2			848	1	1.3	0.0017	0	
1.0715	TLEEITGYLY	10	c-ERB2			402	1	1.1	0	0	
1.0737	YVHAGVCSPY	10	c-ERB2			772	1	1.1	0.010	0.012	0
1.0764	CTPTAKMFNY	10	c-ERB2			1209	1	0.063			
1.0705	LIQRNPQLCY	10	c-ERB2			154	1	0.030			
1.0693	VVQGNLELTY	10	c-ERB2			55	1	0.018			
1.0756	MGDLVDAEEY	10	c-ERB2			1014	1	0.012			
1.1026	KIRKYTMRR	9	c-ERB2			661	3.11		0.76	0.0018	
1.1027	VVFGILIKR	9	c-ERB2			849	3.11		0.11	0.72	
1.0344	LVKSPNHVK	9	c-ERB2			852	3.11		0.48	0.070	
1.0335	VLRENTSPK	9	c-ERB2			754	3.11		0.40	0.013	
1.0329	ILIKRUQQK	9	c-ERB2			673	3.11		0.38	0.0097	
1.0311	ILWKDIFHK	9	c-ERB2			167	3.11		0.28	0.31	
1.1033	KITQIIGLAR	9	c-ERB2			860	3.11		0.17	0.34	
1.0050	GVVFGILIK	9	c-ERB2			848	3.11		0.0057	0.089	
1.0299	QVCTGTDMK	9	c-ERB2			24	3.11		0.0097	0.082	
1.1031	LLDHVRENH	9	c-ERB2			806	3.11		0.0057	<0.0006	
1.1026	CVNCSQFLR	9	c-ERB2			528	3.11		0.0015	0.031	
1.1023	TVCAGGCAR	9	c-ERB2			218	3.11		0.0004	0.023	
1.0331	ILKETELRK	9	c-ERB2			714	3.11		0.019	0.0023	
1.1024	VTAEDCTGR	9	c-ERB2			322	3.11		<0.0002	0.014	
1.0026	DLSYMPIWK	9	c-ERB2			607	3.11		0.0005	0.010	
1.0707	TILWKDIFHK	10	c-ERB2			166	3.11		0.043	3.6	
1.0712	GTQRCEKCSK	10	c-ERB2			227	3.11		0.021	0.61	
1.0256	KVLRENTSPK	10	c-ERB2			753	3.11		0.38	0.22	
1.0702	QLRSLTELK	10	c-ERB2			141	3.11		0.20	0.053	
1.1142	RLVHRDLAAR	10	c-ERB2			840	3.11		0.18	0	
1.0741	LLNWCMQIAK	10	c-ERB2			822	3.11		0.14	0.14	
1.0752	TIDVYMIMVK	10	c-ERB2			948	3.11		0.013	0.12	
1.0721	RILKETELRK	10	c-ERB2			713	3.11		0.0057	0.11	
1.0745	VLVKSPNHVK	10	c-ERB2			851	3.11		0.0057	0.11	
1.1121	SVRQNLQVIR	10	c-ERB2			891	3.11		0.082	0.097	
1.1133	HTVPWDQLFR	10	c-ERB2			429	3.11		0.017	0.075	
1.1127	ILKGGVLIQR	10	c-ERB2			478	3.11		0.0005	0.072	
1.1143	LVSEFSRMAR	10	c-ERB2			148	3.11		0.040	0.0005	
1.1136	GVVFGILIKR	10	c-ERB2			972	3.11		0.0072	0.033	
1.0726	CVARCPSGVK	10	c-ERB2			668	3.11		0.016	0.033	
1.1137	VVFGILIGR	10	c-ERB2			596	3.11		0.022	0.0042	
1.0728	CILRGRQQK	10	c-ERB2			668	3.11		0.0030	0.016	
1.1129	RTVCAGGCAR	10	c-ERB2			672	3.11		0.015	0.0014	
1.1134	GLACHQLCAR	10	c-ERB2			217	3.11		0.0048	0.013	
1.1139	KIPVA1KVLR	10	c-ERB2			508	3.11		0.011	0	
						767	3.11		0.0009	0.099	

Table 23 (a)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0291	VGEADYFEY	9	EBNA1			408	1	0.016			
1.0295	PLRESIVCY	9	EBNA1			553	1	0.010			
1.0681	PVCEADYFEY	10	EBNA1			408	1	0.015			
1.0683	GTWVAGVFVY	10	EBNA1			501	1	0.014			
1.0293	GVFVYGGSK	9	EBNA1			506	3.11		0.30	0.41	
1.1016	KTSLYNLER	9	EBNA1			514	3.11		0.31	0.12	
1.0297	AIKDLVMTK	9	EBNA1			578	3.11		0.068	0.024	
1.0687	QTHIFAEVLK	10	EBNA1			567	3.11		0.010	0.21	
1.1124	GTALAIPQCR	10	EBNA1			523	3.11		0.0028	0.056	

Table 23(b)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
5.0005	CTELKLSDY	9	FLU	A	NP	44	1	3.6			
5.0006	STLELRSRY	9	FLU	A	NP	377	1	0.020			
5.0044	ILRGSVAHK	9	FLU	A	NP	265	3		1.5	0.0057	
5.0051	RMCNILKGK	9	FLU	A	NP	221	3		0.27	0.062	
5.0046	LMQGSTLPR	9	FLU	A	NP	146	3		0.031	0.10	
5.0048	MIDGGRFY	9	FLU	A	NP	32	3		0.059	0.0010	
5.0049	MVLSAFDEK	9	FLU	A	NP	66	3		0.0016	0.061	
5.0054	YIQMCTELK	9	FLU	A	NP	40	3		0.0031	0.090	
5.0042	CINDRNFWR	9	FLU	A	NP	200	3		0.0028	0.024	
5.0104	SLMQGSTLPR	10	FLU	A	NP	145	3		0.12	0.84	
5.0095	KMIDGGRFY	10	FLU	A	NP	31	3		0.50	0.0079	
5.0096	LILRGSVAHK	10	FLU	A	NP	264	3		0.36	0.037	
5.0102	RSGAAGAAVK	10	FLU	A	NP	175	3		0.019	0.0046	
5.0105	SSTLELRSRY	10	FLU	A	NP	376	3		0.0018	0.016	
5.0103	RSRYWAIRTR	10	FLU	A	NP	382	3		0.012	0	
5.0101	KMVLSAFDEK	10	FLU	A	NP	65	3		0.0014	0.010	
5.0061	FYIQMCTEL	9	FLU	A	NP	39	24				2.9
5.0060	AYERMCNIL	9	FLU	A	NP	218	24				0.031
5.0112	RFYIQMCTEL	10	FLU	A	NP	38	24				0.15

Table 23(c)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0155	LLDTASALY	9	HBV	adr	CORE	420	1	25	0.0007	0	
1.0186	SLDVSAAFY	9	HBV	adr	POL	1071	1	17.2	0.0057	0.0006	
2.0125	FTTGRTSLY	9	HBV	ALL		1,342	1	1.3	0.0008	0	
2.0126	MSTTDLEAY	9	HBV	adr		1,521	1	0.85	<0.0008	0	
1.0208	FTTGRTSLY	9	HBV	adr	POL	1,342	1	0.77	0	0	
1.0387	LTKQYLNLY	9	HBV	adw	POL	1280	1	0.50	0.0005	0.0075	
2.0122	LTKQYLNLY	9	HBV	adw		1,280	1	0.095			
1.0146	KVGNFTGLY	9	HBV	adr	POL	629	1	0.068			
2.0227	MSFTDLEAY	9	HBV	adw		1,550	1	0.067			
2.0120	PSQPSRGNY	9	HBV	ayw		984	1	0.057			
2.0112	PSWAFAACY	9	HBV	adw		316	1	0.054			
2.0119	QSAVRKEAY	9	HBV	adw		881	1	0.025			
1.0174	PLDKGIKPY	9	HBV	adr	POL	698	1	0.019			
1.0378	SLMLLYKTY	9	HBV	adr	POL	1092	1	0.017			
2.0115	ASEDLVVSY	9	HBV	ayw		499	1	0.013			
2.0124	PSRGRLGLY	9	HBV	adr/adw		1,364	1	0.011			
2.0121	SISRNINY	9	HBV	adr		1,036	1	0.0097			
1.0519	DLLDTASALY	10	HBV	adr	CORE	419	1	11.1	0	0	
1.0513	LLDFKVRGLY	10	HBV	adr	ENV	120	1	6.3	0.17	0	
2.0239	LSLDVSAAFY	10	HBV	ALL-		1,070	1	4.2	<0.0009	0.0027	
1.0911	FLCCQYLNLY	10	HBV	adr	POL	1250	1	1.1	0.014	0.0048	0.0017
2.0216	QTFGRKLHLY	10	HBV	ayw	POL	1087	1	1.1	0.0056	0.012	
2.0244	KTYGRKLHLY	10	HBV	adw		1,088	1	0.69	0.59	0.22	0
1.0791	KTYGRKLHLY	10	HBV	adw	POL	1088	1	0.57	0.53	0.35	0.0003
2.0242	QTFGRKLHLY	10	HBV	adw		1,067	1	0.37	0.0037	0.011	
1.0554	KTFGRKLHLY	10	HBV	adr	POL	1069	1	0.34	0.294	0.090	0
2.0241	KTFGRKLHLY	10	HBV	adr		1,069	1	0.30	0.15	0.095	0
1.0766	LQDFRVRALY	10	HBV	adw	ENV	120	1	0.21	0.014	0	
1.0826	TTPAQCISMY	10	HBV	adw	ENV	288	1	0.20	0	0	
2.0240	LSISRNINY	10	HBV	adr		1,035	1	0.20	<0.0009	0	
1.0841	PLDKGIKPY	10	HBV	adr	POL	698	1	0.16	0	0	
2.0238	HSASFCCSPY	10	HBV	ayw		767	1	0.15	0.019	0.017	0
1.0765	FLTKQYLNLY	10	HBV	ayw	POL	1279	1	0.12	0	0	
2.0237	RSASFCCSPY	10	HBV	adr/adw		738	1	0.11	0.033	0.020	0
1.0274	WLWGMGISPY	10	HBV	adr	CORE	416	1	0.081			
2.0233	TTPAQCISMY	10	HBV	ayw		288	1	0.066			
1.0542	HTLWKAGILY	10	HBV	adr	POL	714	1	0.030			
2.0231	TSCFRCFGY	10	HBV	adr		226	1	0.018			
2.0246	KSVQHLLSLY	10	HBV	adw		1,161	1	0.016			
1.0910	NLNVSLLLY	10	HBV	adw	POL	1099	1	0.015			
1.0601	WMMWVWGPSL	10	HBV	adr	ENV	359	2.1	0.0029	0.019	0	0.0005
2.0089	LLYQTFGRK	9	HBV	ayw	POL	1084	3		1.8	0.44	
2.0114	IMPARFYPK	9	HBV	ayw		713	3		0.99	1.5	
2.0227	CLHQSPVRK	9	HBV	ayw	POL	867	3		0.14	0.025	
5.0056	SARCSVVRR	9	HBV		POL	531	3		<0.0003	0.567	
2.0077	HLHQOIIKK	9	HBV		POL	686	3		0.041	0.0075	
2.0219	SLPQEHIIQK	10	HBV	ayw	POL	1197	3		0.36	4.2	
2.0234	SMFPSCCCTK	10	HBV	adr/adw		295	3		0.43	1.9	
2.0235	SMFPSCCCTK	10	HBV	ayw		295	3		1.1	1.79	
5.0107	QAFPSPFYK	10	HBV		POL	665	3		0.15	1.3	
2.0214	LLLYQTFGRK	10	HBV	ayw	POL	1083	3		0.89	0.021	
2.0245	YMDDVVLGAK	10	HBV	ALL		1,123	3		0.16	0.0076	
5.0108	TSAICSVVRR	10	HBV		POL	530	3		0.0006	0.013	
2.0228	PYFKAFLCK	9	HBV	ayw	POL	1263	11		0.830	0.085	
2.0268	FTDLEAYFK	9	HBV	adw	"X"	1553	11		0.0002	0.016	
2.0061	KYTSFPWLL	9	HBV	ALL		1,330	24				3.6
2.0059	LYAAVTNFL	9	HBV	adw		1,169	24				3.2
2.0046	FYPNLTKYL	9	HBV	adr		689	24				2.1
2.0045	LYSSTVPSF	9	HBV	adw/ayw		665	24				1.9
2.0048	FYPNVTKYL	9	HBV	ayw		718	24				1.7
2.0049	FYPNVTKYL	9	HBV	adw		718	24				1.6
2.0039	LYSILSPFL	9	HBV	ayw		368	24				0.50
2.0044	LYSSTVPVL	9	HBV	adr		636	24				0.37
2.0038	LYNILSPFL	9	HBV	adr		368	24				0.34
2.0051	NYRVSWPKF	9	HBV	ayw		991	24				0.18
2.0050	HYPQTRHYL	9	HBV	adw/ayw		743	24				0.15
2.0047	HYFKTRHYL	9	HBV	adr		714	24				0.057
2.0060	CYFALMPLY	9	HBV	ALL		1,224	24				0.049
5.0062	AYRPPNAPI	9	HBV		NUC:XN/UCFUS	1.31	24				0.026
2.0054	LYQTFCRKL	9	HBV	ayw		1,085	24				0.016
2.0043	SYQHFRRLL	9	HBV	ayw		607	24				0.011
2.0181	LYSHPIILCF	10	HBV	ALL		1,077	24				1.1
2.0182	LYAAVTNFLL	10	HBV	adw		1,169	24				0.32
2.0188	LYRPLLSLFF	10	HBV	adr		1,371	24				0.25

Table 23(d)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
2.0174	SYQHFRKLLL	10	HBV	ayw		607	24				0.16
2.0173	SYQHFRKLLL	10	HBV	adr/adw		578	24				0.066
2.0176	YYPEHLVNHY	10	HBV	ayw		735	24				0.040
2.0172	AYRPNAPIL	10	HBV	ALL		521	24				0.022
2.0171	GYRWMCLRRF	10	HBV	ALL		234	24				0.011
5.0115	NFLLSLGIHL	10	HBV		POL	572	24				0.0099
1.0377	YVSLMLLYK	9	HBV	adw	POL	1090	3.11		0.31	7.4	
1.0189	LLYKTPGRK	9	HBV	adr	POL	1066	3.11		5.0	0.30	
1.0279	LLYKTYGRK	9	HBV	adw	POL	1095	3.11		2.5	0.40	
1.2350	VTKYLPLDK	9	HBV	adw	POL	722	3.11		0.014	1.3	
1.0176	RHYLHTLWK	9	HBV	adr	POL	719	3.11		1.2	0.070	
1.2367	STVFSFNPK	9	HBV	adr	POL	668	3.11		0.021	0.93	
1.0215	TTDLEAYFK	9	HBV	adr	"X"	1323	3.11		0.0006	0.92	
1.0848	YVSLLLLYK	9	HBV	adr	POL	1061	3.11		0.39	0.92	
1.0383	FTYKAPLTK	9	HBV	adw	POL	1274	3.11		0.17	0.71	
1.0987	HLYPVARQR	9	HBV	adr	POL	1297	3.11		0.54	0.0200	
1.0358	STNRQLGRK	9	HBV	adr	ENV	85	3.11		0.51	0.34	
1.0991	ALRFTSARR	9	HBV	adr	"X"	1488	3.11		0.44	<0.0005	
1.0197	FVNRPEDWK	9	HBV	adr	POL	1197	3.11		0.080	0.41	
1.0269	TVNEKRRLK	9	HBV	adw	POL	703	3.11		0.016	0.40	
1.1043	VVNHYPGTK	9	HBV	adw	POL	740	3.11		0.030	0.35	
1.0352	STTSTGPCK	9	HBV	adr	ENV	277	3.11		0.011	0.29	
1.0213	QVLPKLLHK	9	HBV	adr	"X"	1505	3.11		0.10	0.28	
1.0272	LTKYLPLDK	9	HBV	adr	POL	683	3.11		0.0039	0.23	
1.0374	CLHQRALVRK	9	HBV	adr	POL	878	3.11		0.22	0.20	
1.0980	VVDRSQFSR	9	HBV	adr	POL	963	3.11		0.011	0.20	
1.0382	PLYACDQAK	9	HBV	adw	POL	1259	3.11		0.18	0.084	
2.0074	YVNTNMGLK	9	HBV	ayw	CORE	507	3.11		0.16	0.048	
1.0199	FLYACKQSK	9	HBV	adr	POL	1230	3.11		0.11	0.018	
1.0972	RLADEGLNR	9	HBV	adr	POL	601	3.11		0.10	0.035	
1.0976	AVNHYFKTR	9	HBV	adr	POL	711	3.11		0.0017	0.036	
1.0975	RLKLIMPAR	9	HBV	adr	POL	680	3.11		0.095	0.0032	
1.0977	ILYKRETTK	9	HBV	adr	POL	730	3.11		0.095	<0.0005	
1.0993	KVFVLGGCK	9	HBV	adr	"X"	1548	3.11		0.043	0.082	
1.0165	NVSSFHWTHK	9	HBV	adr	POL	621	3.11		0.072	0.076	
1.0982	LLLYKTPGR	9	HBV	adr	POL	1065	3.11		0.072	0.074	
1.0978	RLVFQTSTR	9	HBV	adr	POL	757	3.11		0.066	0.0032	
1.0229	FVLGGCRHK	9	HBV	adr	"X"	1550	3.11		0.065	0.019	
1.1062	RLYLQTSTR	9	HBV	adw	POL	786	3.11		0.064	0.0002	
1.1043	MLLYKTYGR	9	HBV	adw	POL	1094	3.11		0.061	0.0032	
1.0170	TVNEKRRLK	9	HBV	adr	POL	674	3.11		0.048	0.030	
1.1045	NLYPVARQR	9	HBV	adw	POL	1286	3.11		0.042	0.0011	
1.1046	LPYRFPTTGR	9	HBV	adw	POL	1407	3.11		0.021	0	
1.0845	LVSPGVWIR	9	HBV	adr	CORE	509	3.11		0.0033	0.030	
1.0981	LVGSIGLPR	9	HBV	adr	POL	1022	3.11		0.0008	0.015	
1.0967	HISCLTFGR	9	HBV	adr	CORE	494	3.11		0.013	0.011	
1.1067	SVFSRLPDR	9	HBV	adw	POL	1424	3.11		0.0007	0.010	
1.0909	SVFSRLPDR	9	HBV	adr	POL	1395	3.11		0.0004	0.010	
1.0844	TLPQEHIVLK	10	HBV	adr	POL	1179	3.11		0.092	5.6	
1.0205	TVPVFNPHWK	10	HBV	ayw	POL	669	3.11		0.0067	4.2	
1.0543	TLWKAGILYK	10	HBV	adr	POL	724	3.11		3.3	1.2	
1.0407	SMYPSCCCTK	10	HBV	ayw	ENV	295	3.11		1.5	3.4	
1.1153	RLPFRPTTGR	10	HBV	adw	POL	1406	3.11		2.8	0.090	
1.0584	STTDLEAYFK	10	HBV	adr	"X"	1322	3.11		0.0046	2.7	
1.0554	LLLYKTPGRK	10	HBV	adr	POL	1065	3.11		2.5	0.012	
1.0799	TVNAHRNLFK	10	HBV	adw	"X"	1529	3.11		0.82	0.46	
1.0586	EAYFKDCLFK	10	HBV	adr	"X"	1527	3.11		0.037	0.74	
1.1081	LVVDFSQFSR	10	HBV	adr	POL	962	3.11		0.0009	0.63	
1.0799	MLLYKTYGRK	10	HBV	adw	POL	1094	3.11		0.61	0.020	
1.0546	TAYSHLSTSK	10	HBV	adr	POL	858	3.11		0.26	0.092	
1.0562	SLGIHLNPNK	10	HBV	adr	POL	1150	3.11		0.20	0.078	
1.1152	RLGLYRPLLR	10	HBV	adw	POL	1397	3.11		0.19	0.0084	
1.0547	VTGGVFLVDK	10	HBV	adr	POL	943	3.11		0.035	0.17	
1.1150	RIRTRTPTAR	10	HBV	adw	POL	942	3.11		0.17	0.0002	
1.0541	TVNCHQVLPK	10	HBV	adr	"X"	1500	3.11		0.073	0.092	
1.1091	SLPFQPTTGR	10	HBV	adr	POL	1377	3.11		0.077	0.043	
1.1072	TLPETTVVRR	10	HBV	adr	CORE	532	3.11		<0.0003	0.075	
1.1089	GTDNSVVLSR	10	HBV	adr	POL	1320	3.11		0.025	0.072	
1.1071	STLPETTVVR	10	HBV	adr	CORE	531	3.11		0.0005	0.068	
2.0210	KVTKYLPLDK	10	HBV	ayw	POL	721	3.11		0.027	0.053	
1.1148	STRHGDKSFR	10	HBV	adw	POL	792	3.11		0.0057	0.038	
1.0935	VLSCWWLQFR	10	HBV	adw	POL	923	3.11		0.029	0.037	
1.0781	NVTKYLPLDK	10	HBV	adw	POL	721	3.11		<0.0004	0.023	

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.1092	RVCCQLDPAR	10	HBV	adr	X	1422	3.11		0.0019	0.023	
1.0793	SLGIHLNPQK	10	HBV	adw	POL	1179	3.11		0.017	0.014	
1.0909	YLVSFGVWIR	10	HBV	adr	CORE	508	3.11		0.015	0.0027	
2.0207	FVCPLTVNEK	10	HBV	ayw	POL	668	3.11		0.0057	0.015	
1.0535	YVCPLTVNEK	10	HBV	adr	POL	668	3.11		0.0068	0.014	
1.1075	RLADEGLNRR	10	HBV	adr	POL	601	3.11		0.013	0.0004	
1.1086	IVLKLKQCFR	10	HBV	adr	POL	1185	3.11		0.013	0.0024	
1.0773	PIPSSWAFAK	10	HBV	adw	ENV	314	3.11		<0.0003	0.010	
1.0778	LTVNENRRLK	10	HBV	adw	POL	702	3.11		0.0025	0.0095	

Peptide#	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0118	CTCCSSDLY	9	HCV		LORF	1123	1	3.0	0	0.010	
1.0112	NIVDVQYLY	9	HCV		NS1/ENV2	697	1	0.40	0	0.010	
2.0034	VQDCNCSIY	9	HCV			302	1	0.54	0.0005	0.0003	
2.0035	LTPRCMVDY	9	HCV			605	1	0.078			
1.0145	RVCEKMALY	9	HCV		LORF	2588	1	0.053			
1.0140	DVVCCSMSY	9	HCV		LORF	2416	1	0.039			
2.0036	PTIFKIRMY	9	HCV			626	1	0.012			
1.0509	GLSAPSLHSY	10	HCV		LORF	2886	1	0.41	0.013	0.0034	0.0002
1.0489	TLHGPTPLLY	10	HCV		LORF	1617	1	0.30	0.11	0.0024	
2.0037	EYVLLLFLL	9	HCV			719	24				1.4
2.0169	MYVCGVEHRL	10	HCV			633	24				0.026
2.0170	EYVLLLFLLL	10	HCV			719	24				0.010
1.0139	SVPAEILRK	9	HCV		LORF	2269	3.11		0.016	0.87	
1.0955	QLFTPSPRR	9	HCV		ENV1	290	3.11		0.75	0.033	
1.0080	RLGVRATRK	9	HCV		LORF	43	3.11		0.74	0.16	
1.0123	LIPCHSKKK	9	HCV		LORF	1391	3.11		0.54	0.19	
1.0122	HLIPCHSKK	9	HCV		LORF	1390	3.11		0.25	0.010	
1.0952	KTSERSQPR	9	HCV		CORE	51	3.11		0.16	0.084	
1.0120	AVCTRGVAK	9	HCV		LORF	1183	3.11		0.016	0.038	
1.0143	EVPCVQPEK	9	HCV		LORF	2543	3.11		0.0019	0.013	
1.0137	ITRVESENK	9	HCV		LORF	2261	3.11		0.015	0.0079	
1.0957	CGTSLTGR	9	HCV		LORF	1042	3.11		0.0095	0.011	
1.0496	GVAGALVAFK	10	HCV		LORF	1858	3.11		0.87	1.1	
1.0480	HLHAPTCSGCK	10	HCV		LORF	1227	3.11		0.57	0.0051	
1.1062	RMYVCGVEHR	10	HCV		NS1/ENV2	632	3.11		0.27	0.012	
1.0485	HLIPCHSKKK	10	HCV		LORF	1390	3.11		0.27	0.025	
1.0484	TLGPGAYMSK	10	HCV		LORF	1261	3.11		0.17	0.13	
1.0067	CVGIYLLPNR	10	HCV		LORF	3002	3.11		0.0029	0.032	
1.1063	LLFLLLLADAR	10	HCV		NS1/ENV2	725	3.11		0.015	0	

Table 23(e)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0014	FRDYVDRFY	9	HIV		GAG	298	1	0.090			
2.0129	IYQYMDDLY	9	HIV			875	1	0.064			
1.0028	TVLDVGDAY	9	HIV		POL	802	1	0.018			
1.0412	VTVLDVGDAY	10	HIV		POL	801	1	0.28	0	0.0004	
1.0415	VIYQYMDDLY	10	HIV		POL	874	1	0.25	0.0027	0.0090	
2.0252	VTVLDVGDAY	10	HIV			801	1	0.088			
1.0431	EVNIVTDSQY	10	HIV		POL	1187	1	0.053			
1.0441	LVAVHVASGY	10	HIV		POL	1329	1	0.039			
1.0442	PAETGQETAY	10	HIV		POL	1345	1	0.013			
2.0251	ISKIGPENPY	10	HIV			742	1	0.013			
2.0255	QMAVRHNFK	10	HIV			1,432	3		0.61	0.44	
2.0264	RYLKDQQLL	9	HIV			2,778	24				0.76
2.0134	RYLKDQQLL	9	HIV			2,778	24				0.32
2.0065	TYQYQEFF	9	HIV			1,033	24				0.30
2.0131	TYQYQEFF	9	HIV			1,033	24				0.20
2.0063	IYQFPRNL	9	HIV			1,036	24				0.052
2.0132	IYQFPRNL	9	HIV			1,036	24				0.033
2.0064	IYQYMDDLY	9	HIV			875	24				0.013
2.0067	IYKRWIILGL	10	HIV			366	24				0.017
2.0190	IYKRWIILGL	10	HIV			366	24				0.014
2.0249	LYPLASLRSL	10	HIV			506	24				0.014
1.0369	KLAGRHWPVK	9	HIV		POL	1358	3.11		2.7	0.049	
1.0944	AVRHNFRQK	9	HIV		POL	1434	3.11		0.17	1.8	
1.0032	AIFQSSMTK	9	HIV		POL	853	3.11		1.1	0.96	
1.0046	IVIWGKTPK	9	HIV		POL	1075	3.11		0.085	0.37	
1.0379	KLTEDRWNK	9	HIV		VIF	1712	3.11		0.013	0.27	
1.0027	CIPHPAGLK	9	HIV		POL	788	3.11		0.23	0.065	
1.0359	QIIEQLIKK	9	HIV		POL	1215	3.11		0.0091	0.16	
1.0939	KIWFSYKGR	9	HIV		GAG	443	3.11		0.12	0.0005	
1.0072	ILATDIQTK	9	HIV		POL	1458	3.11		0.025	0.098	
1.0036	MGYELHPDK	9	HIV		POL	925	3.11		0.064	0.096	
1.0363	YLAWVPAHK	9	HIV		POL	1227	3.11		0.077	0.057	
1.0938	KIWPSHKGR	9	HIV		GAG	443	3.11		0.077	<0.0005	
1.0047	FVNTPPLVK	9	HIV		POL	1111	3.11		0.012	0.066	
1.0024	NTPVFAIKK	9	HIV		POL	752	3.11		0.033	0.060	
1.0380	TVQCTHGIK	9	HIV		ENV	2420	3.11		0.0021	0.046	
1.0013	ILDIRQGPK	9	HIV		GAG	287	3.11		0.062	0.0048	
1.0015	RDYVDRFYK	9	HIV		GAG	299	3.11		0.0007	0.040	
1.0258	GIGQAFPIK	9	HIV		POL	1199	3.11		<0.0008	0.040	
1.0264	VLRLDGDIK	9	HIV		POL	1254	3.11		0.038	0.032	
1.0236	LVDFRELNK	9	HIV		POL	749	3.11		0.011	0.030	
1.0378	KVVFRRKAK	9	HIV		POL	1513	3.11		0.029	0.0029	
1.0942	MTIGLEFFR	9	HIV		POL	839	3.11		<0.0008	0.016	
1.0463	TVYYGVPVWK	10	HIV		ENV	2185	3.11		3.8	7.8	
1.0418	TVQFRVLPEK	10	HIV		POL	935	3.11		0.16	5.6	
1.0447	AVRHNFPKK	10	HIV		POL	1434	3.11		0.66	0.85	
1.0437	KVLRLDGIDK	10	HIV		POL	1253	3.11		0.56	0.78	
1.0408	KLVDFRELNK	10	HIV		POL	748	3.11		0.51	0.090	
1.0403	KLKPGMDGPK	10	HIV		POL	706	3.11		0.39	0.076	
1.2895	FLGKIWPSYK	10	HIV		GAG	440	3.11		0.32	0.024	
1.1054	KIGKIWPSYYK	10	HIV		POL	1474	3.11		0.033	0.21	
1.0410	CIPHPAGLLK	10	HIV		POL	788	3.11		0.051	0.17	
1.0436	LVKLWYQLEK	10	HIV		POL	1117	3.11		0.056	0.082	
1.0398	MIGGIGGFIK	10	HIV		POL	642	3.11		0.0099	0.055	
1.0413	MTIGLEFFR	10	HIV		POL	839	3.11		0.015	0.038	
1.0453	VVIGRNLLQK	10	HIV		POL	1504	3.11		<0.0005	0.021	
1.0394	FLGKIWPSHK	10	HIV		GAG	440	3.11		0.030	0.0013	
1.1059	IVQQQNNLLR	10	HIV		ENV	2741	3.11		0.0024	0.019	
1.0417	FTTPDKKHQK	10	HIV		POL	909	3.11		<0.0002	0.015	
1.0405	LVEKTEMEK	10	HIV		POL	729	3.11		0.0002	0.012	
1.0392	LVQNANPDCK	10	HIV		GAG	327	3.11		<0.0002	0.011	

Table 23(f)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0225	ISEYRHYCY	9	HPV	16	E6	80	1	7.3	0.0011	0.004	
1.0230	QAEPDRAHY	9	HPV	16	E7	44	1	0.021			
1.0610	LQDIETCVY	10	HPV	18	E6	25	1	0.25	0.0056	0.012	
2.0159	YSKISEYRHY	10	HPV	16	E6	77	1	0.17	<0.0009	0	
2.0162	YSKISEYRHY	10	HPV	16	E6	77	1	0.11	<0.0009	0	
1.0999	HCDTPTLHEY	10	HPV	16	E7	2	1	0.087			
1.0601	QPETTDLYCY	10	HPV	16	E7	16	1	0.033			
1.0913	IHDIILECVY	10	HPV	16	E6	30	1	0.032			
2.0140	YSRIRELRHY	10	HPV	18	E6	72	1	0.018			
2.0164	YSRIRELRHY	10	HPV	18	E6	72	1	0.012			
1.0994	AVCDKCLJCFY	10	HPV	16	E6	68	1	0.0095			
2.0161	LLRCLRCQK	10	HPV	18	E6	101	3		0.081	0.078	
2.0032	HTMLCMCCKX	9	HPV	18	E7	59	11		0.020	0.079	
2.0029	VYCKTVLEL	9	HPV	18	E6	33	24				0.33
2.0027	CVSLVGTTL	9	HPV	16	E5	87	24				0.057
2.0024	VYDFARFDL	9	HPV	16	E6	49	24				0.032
2.0031	LYNLLRCL	9	HPV	18	E6	98	24				0.019
2.0030	VYGDTLEKL	9	HPV	18	E6	85	24				0.010
1.0239	SVYGDTLEK	9	HPV	18	E6	84	3.11		0.39	2.3	
1.0243	SVYGDTLEK	9	HPV	18	E6	84	3.11		0.55	1.1	
1.0244	SVYGDTLEK	9	HPV	18	E6	84	3.11		0.70	0.95	
1.0226	TTLEQQYNK	9	HPV	16	E6	93	3.11		0.010	0.67	
1.0241	SIPHAACHK	9	HPV	18	E6	59	3.11		0.0094	0.25	
1.0227	SIPHAACHK	9	HPV	18	E6	59	3.11		0.017	0.12	
1.0233	IVCPICSQK	9	HPV	16	E7	89	3.11		0.035	0.023	
1.0997	KLEHLNEKR	9	HPV	18	E6	117	3.11		0.025	<0.0005	
1.0234	LIRCLKCQK	9	HPV	18	E6	102	3.11		0.019	0.0012	
1.0653	IILECVYCK	9	HPV	16	E6	33	3.11		0.0016	0.019	
1.0999	CIDFYSRIR	9	HPV	18	E6	68	3.11		0.017	0.0018	
1.0998	CIDFYSRIR	9	HPV	18	E6	68	3.11		0.010	0.0009	
1.0996	GTTLEQQYNK	10	HPV	16	E6	92	3.11		0.010	0.98	
1.0606	LLRCLRCQK	10	HPV	18	E6	101	3.11		0.056	0.39	
1.0998	LLRCLRCQK	10	HPV	18	E6	106	3.11		0.12	0.34	
1.0639	LLRCLRCQK	10	HPV	18	E6	101	3.11		0.16	0.11	
1.0614	LTEVFEFARK	10	HPV	18	E6	43	3.11		0.0029	0.11	
1.0605	GIVCPKSQK	10	HPV	16	E7	88	3.11		0.0017	0.060	
1.0625	LTEVFEFARK	10	HPV	18	E6	43	3.11		0.0012	0.041	
1.0991	DIILECVYCK	10	HPV	16	E6	32	3.11		0.0065	0.021	
1.1101	KLEHLNEKR	10	HPV	18	E6	117	3.11		0.013	0	
1.1095	CVYCKQQLLR	10	HPV	16	E6	37	3.11		0.011	0.0059	

Table 23(g)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
2.0020	EVDPIGHLY	9	MAGE	3		141	1	18	0.0002	0.0009	
3.0172	EADPTSNTY	9	MAGE	5/51		161	1	9.9	0.0006	0.0006	0
1.0256	TQOLVQEKY	9	MAGE	1		240	1	2.1	0	0.0002	
3.0173	EVDPIGHVY	9	MAGE	4		161	1	1.9	<0.0002	<0.0002	0
1.0254	EADPTSNSY	9	MAGE	1		141	1	1.1	0	0	
1.0259	LVQEKYLEY	9	MAGE	1		243	1	0.42	0.0013	0.053	
6.0053	TSYVKVLEY	9	MAGE	1	new	275	1	0.099			
2.0029	SSLPTTMNY	9	MAGE	3		9	1	0.055			
2.0011	CSVVGNWQY	9	MAGE	3		77	1	0.050			
2.0008	SSFITDNY	9	MAGE	2		9	1	0.043			
1.0252	MLESVIKNY	9	MAGE	1		128	1	0.011			
2.0147	ASSLPTTMNY	10	MAGE	3		8	1	2.6	<0.0009	0.013	
2.0167	LTQOLVQEKY	10	MAGE	1		239	1	1.2	<0.0009	0.0073	
6.0114	ETSYVKVLEY	10	MAGE	1	new	274	1	0.56			
2.0141	ASSFITTNY	10	MAGE	2		8	1	0.17	<0.0009	0.026	
1.0648	DLVQEKYLEY	10	MAGE	1		242	1	0.044			
6.0055	TSYVKVLEY	9	MAGE	1	new	275	3		0.71	0.010	
4.0119	TTDNFTRQR	9	MAGE	1		44	3		0.043	0.37	
6.0054	ALAETSYVK	9	MAGE	1	new	271	3		0.31	0.36	
4.0132	LTQOLVQEK	9	MAGE	1		239	3		<0.0003	0.14	
6.0052	LVQEKYLEY	9	MAGE	1	new	243	3		0.0026	0.024	
4.0131	HSAYGEPRK	9	MAGE	1		229	3		0.014	0.0009	
4.0122	LFRAVITKK	9	MAGE	1		97	3		0.011	0.005	
4.0124	SVRFFPSLR	10	MAGE	1	new	290	3		0.43	0.0099	
4.0161	ADLVGFLLLK	10	MAGE	1		107	3		0.35	0.29	
4.0160	ESLFRAVITK	10	MAGE	1		95	3		0.14	0.088	
6.0119	DLVQEKYLEY	10	MAGE	1	new	242	3		0.032	0.0051	
6.0123	YVIKVSARVR	10	MAGE	1	new	283	3		0.019	0.0009	
4.0168	LSVMEVYDGR	10	MAGE	1		218	3		<0.0003	0.012	
4.0163	KAEMLESVIK	10	MAGE	1		125	3		<0.0003	0.0097	
6.0125	RALAETSYVK	10	MAGE	1	new	270	11		0.19	0.24	
2.0010	NYPLWSQSY	9	MAGE	3		16	24				0.027
2.0165	NYKHGFPEIF	10	MAGE	1		135	24				0.25
2.0151	LYIFATCLCL	10	MAGE	3		113	24				0.048
6.0126	SYVKVLEYVI	10	MAGE	1	new	276	24				0.036
1.0248	SLFRAVITK	9	MAGE	1		96	3.11		4.1	2.7	
1.1006	SVMEVYDGR	9	MAGE	1		219	3.11		0.0080	1.3	
1.1004	TTDNFTRQR	9	MAGE	1		44	3.11		0.016	1.0	
1.0257	LTQOLVQEK	9	MAGE	1		239	3.11		0.0002	0.38	
1.0634	SLFRAVITK	10	MAGE	1		96	3.11		1.2	0.98	
1.0647	LLTQDLVQEK	10	MAGE	1		239	3.11		0.0004	0.16	
1.0440	MLESVIKNYK	10	MAGE	1		128	3.11		0.14	0.027	
1.0644	LLGDNQMPK	10	MAGE	1/3		162	3.11		0.020	0.011	
1.0630	SLEQRSLHCX	10	MAGE	1		2	3.11		0.015	0.015	

Table 23(h)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0281	CSDCTTIHY	9	p53			226	1	29.5	0.0010	0.029	
1.0667	CTAKSVTCTY	10	p53			117	1	0.33	0.023	0.049	0
1.0672	RVEGNLRVEY	10	p53			196	1	0.022			
1.0278	RVRAMAIYK	9	p53			156	3.11		1.5	0.73	
1.0276	CTYSPALNK	9	p53			124	3.11		0.46	1.1	
1.0285	NTSSSFQPK	9	p53			311	3.11		0.0009	0.095	
1.0284	RTEEENLRK	9	p53			283	3.11		0.0015	0.091	
1.0287	ELNEALELK	9	p53			343	3.11		0.020	0.0052	
1.0678	RTEEENLRKK	10	p53			283	2.11		2.3	0.0080	
1.1113	KTYQGSYGFR	10	p53			101	3.11		2.6	0.88	
1.1115	VVRRCPHHER	10	p53			172	3.11		0.099	0.0017	
1.0679	NTSSSFQPKK	10	p53			311	3.11		0.0035	0.054	
1.1121	RVCACPGRDR	10	p53			273	3.11		0.014	0.011	
1.1116	GLAPPQHLIR	10	p53			187	2.11		0.013	0.0006	

Table 23(i)

114

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
3.0175	KCEYFVEMY	9	PAP			322	1	3.4	<0.0002	0.0002	0
3.0174	LCEYRKKEY	9	PAP			81	1	0.76	<0.0002	0.0002	0
3.0146	ASCHLTELY	9	PAP			311	1	0.77	<0.0002	0.055	0
3.0143	ESYICHEQVY	9	PAP			95	1	0.096	<0.0002	0.0002	0
3.0227	LSELSLLSLY	10	PAP			238	1	14	0.0028	0.0004	0
3.0235	LSELSLLSLY	10	PAP			238	1	12	0.0005	0.0004	0
3.0236	LTQLCMEQHY	10	PAP			70	1	0.62	0.015	0.0024	0.0022
3.0238	KCEYFVEMY	10	PAP			322	1	0.018			
3.0230	LVNEILNHMK	10	PAP			263	3		0.056	0.12	
3.0158	ATQPSYKK	9	PAP			274	11		0.10	1.2	
3.0231	ETLKSEEFQK	10	PAP			170	11		<0.0004	0.014	
3.0161	LYFEKCEYF	9	PAP			318	24				2.5
3.0160	LYCESVHNF	9	PAP			213	24				0.44
3.0159	PYKDFRATL	9	PAP			183	24				0.11
3.0162	VYNCLLFPY	9	PAP			302	24				0.032
3.0232	PYASCHLTEL	10	PAP			309	24				0.024

Table 23(j)

115

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0270	ALPEKPSLY	9	PSA			231	1	0.011			
2.0157	VSHSFPHPLY	10	PSA			88	1	0.15	<0.0003	0.0015	
1.0265	PLYDMSLLK	9	PSA			95	3.11		0.24	0.007	
1.0273	VVHYRKWIK	9	PSA			242	3.11		0.0072	0.093	
1.0272	YTKVVHYRK	9	PSA			239	3.11		0.0006	0.068	
1.1009	SLLKNRFLR	9	PSA			100	3.11		0.0024	0.047	
1.0260	IVGGWECEK	9	PSA			21	3.11		0.041	0.019	
1.0269	QVHPQKVTK	9	PSA			182	3.11		0.0060	0.014	
1.1112	SLYTKVVHYR	10	PSA			237	3.11		0.28	0.23	
1.0653	LTAAHCIRNK	10	PSA			57	3.11		0.14	0.083	
1.0651	RIVGGWECEK	10	PSA			20	3.11		0.066	0.067	
1.0662	KVVHYRKWIK	10	PSA			241	3.11		0.065	0.065	
1.1111	VTKFMLCAGR	10	PSA			188	3.11		0.0003	0.012	
3.0108	MLLRLSEPA	9	PSA			118	Random				

Table 23 (k)

TABLE 24: CTL EPITOPES IDENTIFIED IN PEPTIDE SCREENING.

Sequence	Antigen	Motif	Id
EVDPIGHLY	MAGE3	A01	1044.07
ASSLPTTMNY *	MAGE3	A01	1044.01
EADPTGHSY	MAGE1	A01	958.01
SSLPTTMNY *	MAGE3	A01	1072.02 *
GSVVGNWQY *	MAGE3	A01	1072.03 *
ALAETSYVK *	MAGEIN	A03	1072.38 *
SLFRAVITK	MAGE1	A11	1072.13
RALAETSYVK	MAGEIN	A11	1072.39
ESLFRAVITK	MAGE1	A11	1072.15
KVYLAMVPAHK	MAGEI	A3/11 *	1069.42 *
TVYYGVPVWK	HIV	A03	1069.43
KLAGRWPVK	HIV	A03	1069.44
KMIGGIGGFIK	HIV	A03	1069.45
AIFQSSMTK	HIV	A03	966.01
WTYQIYQEPFK	HIV	A03	1069.46
FLGKIWPSHK *	HIV	A03	1069.56 *
TVYYGVPVWK	HIV	A11	1052.03
VTVYYGVPVWK	HIV	A11	1069.47
GVAGALVAFK	HCV	A03	1073.10
CTCGSSDLY	HCV	A11	1069.62
GVAGALVAFK	HCV	A01	1052.05
LLDTASALY *	HBV	A01	1069.01 *
TLMKAGILYK	HBV	A03	1069.15

* borderline positive

Table 25 a
Peptides Synthesized
by Cytel For Loading
Onto Acid Stripped
Autologous PBMCs and
PHA Blasts

Peptide ID #	Antigen	Sequence
777.03	HBs	FLLTRILTI
924.07	HBc	FLPSDFFPSV
927.32	HBp	GLYSSTVPV
938.01	MAGE 1	EADPTGHSY
939.03	PSA	VLVHPQWVL
941.01	HBc	FLPSDYFPSV
1044.04	PAP	ILLWDPIPV
1044.05	PSA	KLQCVDLVHI
1044.06	PSA	MLLRLSEPAEL

Table 25 b

Cell Population	^{125}I-Labeled Peptide +/- Cold Peptide	CPMS +/- std. dev.	
JY acid stripped	- cold peptide	3553 ± 157	n = 3
JY acid stripped	+ cold peptide	13	n = 1
JY control	-cold peptide	370 ± 37	n = 3
JY control	+ cold peptide	50	n = 1

WHAT IS CLAIMED IS:

1.   A composition comprising an immunogenic peptide having a HLA-A3.2 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue selected from the group consisting of L, M, I, V, S, A, T, F, C, G, D and E;

and a second conserved residue of K, R, Y, H and F;

wherein the first and second conserved residues are separated by 6 to 7 residues.

2.   The composition of claim 1, wherein the first conserved residue is at the second position from the N-terminus.

3.   A composition comprising an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and

a second conserved residue of D, E, A, S and T;

a third conserved residue of Y;

wherein the first and second conserved residues are adjacent and the second and third conserved residues are separated by 5 or 6 residues.

4.   The composition of claim 3, wherein the first conserved residue is at the second position from the N-terminus.

5.   A composition comprising an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and

a second conserved residue of Y;

wherein the first and second conserved residues are separated by 6 to 7 residues.

6.   The composition of claim 5, wherein the first conserved residue is at the second position from the N-terminus and the second conserved residue is at the ninth or tenth position from the N-terminus.

7.   A composition comprising an immunogenic peptide having an HLA-A1 binding motif, which immunogenic peptide has between 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus.

a first conserved residue of D, E, A, S and T; and

a second conserved residue of Y;

wherein the first and second conserved residues are separated by 5 to 6 residues.

8.   The composition of claim 5, wherein the first conserved residue is at the third position from the N-terminus and the second conserved residue is at the ninth or tenth position from the N-terminus.

9.   A composition comprising an immunogenic peptide having a HLA-A11 binding motif, which peptide has between about 9 and about 10 residues and the following residues, from the Nterminus to the C-terminus:

a first conserved residue of L, M, I, V, A, S, T, G, N, Q, C, F, D, E; and

a second conserved residue of K, R, H;

wherein the first and second conserved residues are separated by 6 to 7 residues.

10.   The composition of claim 9, wherein the first conserved residue is at the second position from the N-terminus.

11.   A composition comprising an immunogenic peptide having a HLA-A24.1 binding motif, which immunogenic peptide

120

has between about 9 and about 10 residues and the following
residues, from the N-terminus to the C-terminus:
          a first conserved residue of Y, F, W; and
          a second conserved residue of F, I, L, W, M;
          wherein the first and second conserved residues are
separated by 6 to 7 residues.

          12. The composition of claim 11, wherein the first
conserved residue is at the second position from the
N-terminus.

          13. A composition comprising an immunogenic peptide
having an HLA-A3.2 binding motif, which immunogenic peptide
has 9 or 10 residues:
          a first conserved residue at the second position
selected from the group consisting of A, I, L, M, T, and V;
and a second conserved residue at the C terminal postion
selected from the group consisting of K and R.
          wherein the first and second conserved residues are
separated by 6 to 7 residues.

          14. A composition comprising an immunogenic peptide
having an HLA-A11 binding motif, which immunogenic peptide has
9 or 10 residues and the following residues, from the
N-terminus to the Cterminus:
          a first conserved residue at the second position from
the N terminus selected from the group consisting of A, I, L,
M, T and V; and
          a second conserved residue at the C terminal position
selected from the group consisting of K;
          wherein the first and second conserved residues are
separated by 6 to 7 residues.

          15. A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and an immunogenic peptide
having a HLA-A3.2 binding motif, which immunogenic peptide has
between about 9 and about 10 residues and the following
residues, from the N-terminus to the C-terminus:

121

a first conserved residue selected from the group consisting of L, M, I, V, S, A, T, F, C, G, D and E; and

a second conserved residue of K, R and Y;

wherein the first and second conserved residues are separated by 6 to 7 residues.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and

a second conserved residue of D, E, A, S and T;

a third conserved residue of Y;

wherein the first and second conserved residues are separated by 1 residue and the second and third conserved residues are separated by 5 or 6 residues.

17. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogeric peptide having a HLA-A1 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S or M; and

a second conserved residue of Tyr;

wherein the first and second conserved residues are separated by 6 to 7 residues.

18. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A1 binding motif, which peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of D, E, S, T; and

a second conserved residue of Y;

wherein the first and second conserved residues are separated by 5 to 6 residues.

19.   A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A24.1 binding motif, which peptide has

a first conserved residue of Y, F, W; and

a second conserved residue of F, I, L, W, or m;

wherein the first and second conserved residues are separated by 6 to 7 residues.

20.   A method of identifying an immunogenic peptide comprising the following steps:

determining a binding motif for an MHC molecule encoded by a preselected MHC Class I allele;

screening an amino acid sequence of an antigenic protein for the presence of the binding motif;

selecting a sequence in the antigenic protein having the binding motif;

preparing a test peptide of about 8 and about 11 residues comprising the selected subsequences;

determining the ability of the test peptide to bind to the preselected MHC allele and induce a CTL response, thereby identifying immunogenic peptides.

HLA-A PURIFICATION AND
PEPTIDE ELUTION

Cellular Source: HLA-Antigens   a)   EBV transformed B cell
 (5-10x10⁹ cell equivalents)   lines - homozygous
                                      b)   HLA-A transfectants -
                                           e.g. .221-HLA-A1
                                      c)   P815 transfectants
                                           (mouse mastocytoma)

Detergent Lysis   1% NP-40 or 1% Renex 30 plus
 (10⁸ cells/ml)   protease inhibitors - 1 hr,4°C

Detergent Lysate   Centrifugation at 15,000xg,
                                      30 min.

Affinity Chromatography   mAb-Sepharose   5 mg/ml
                                        5-10 ml column

Purified HLA-A Antigen   Anticipated yields   450-900 µg

Acid Treatment   10% acetic acid, 5 min, 100°C

Peptides   YM3 filtration, 3kD cut-off

Sequence/Motif   D. Hunt - HPLC/EI-TMS
                                      Cytel - HPLC/ABI 477A

4/6/92:RK
                    Figure 1
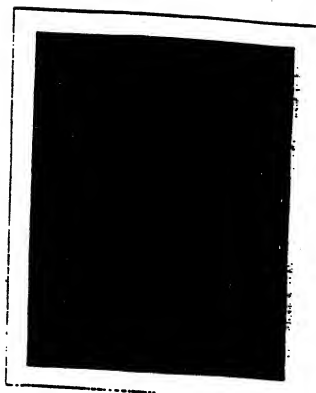
Figure 2

Peptides from HLA A3

RP-HPLC separation of HLA-A3 acid eluted peptides.
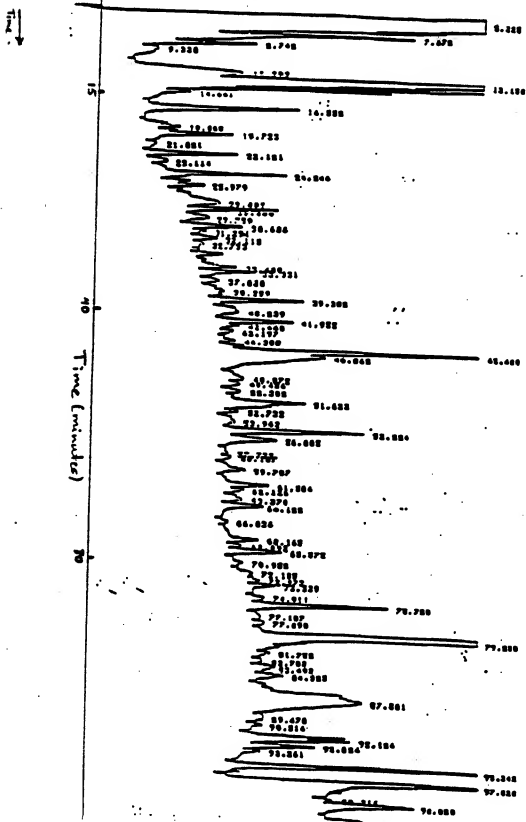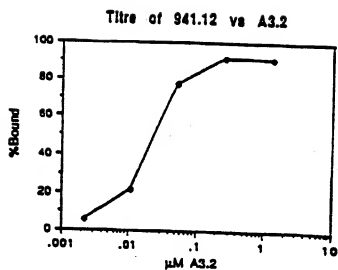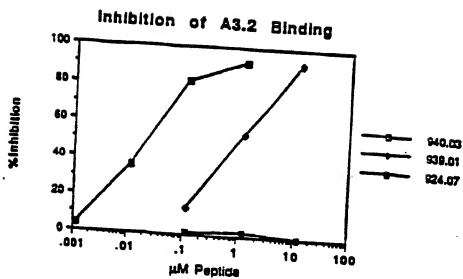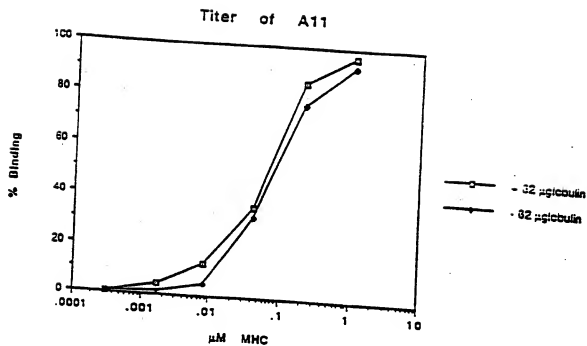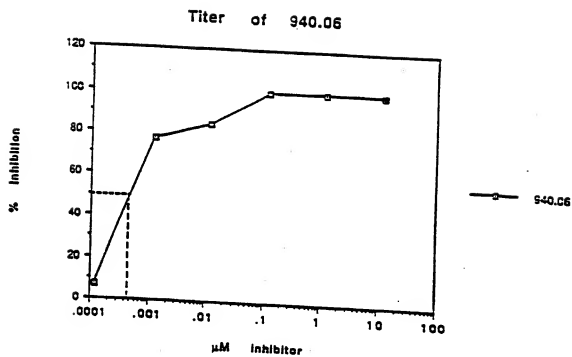
Figure 4

Figure 5
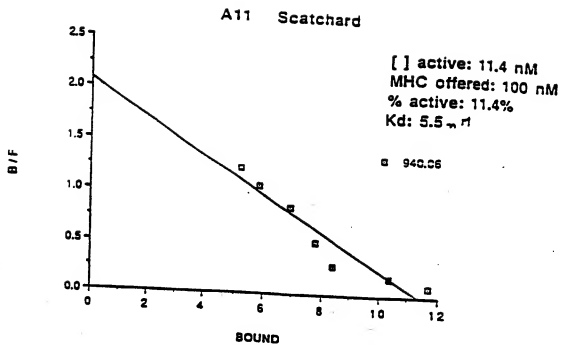
Figure 6

Figure 7

Figure 8

Titer of 944.02 against A1.
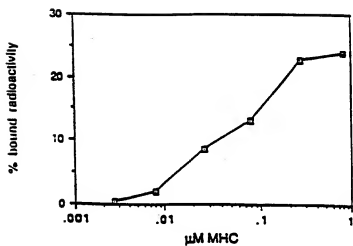
Figure 9

A1 inhibition titration.
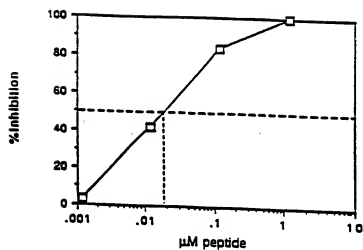
Figure 10

Scatchard for A1 vs. 944.02
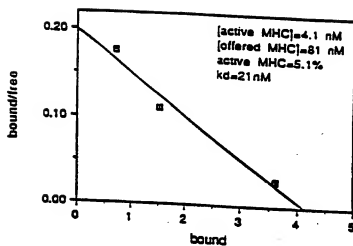
Figure 11

A24 titration.
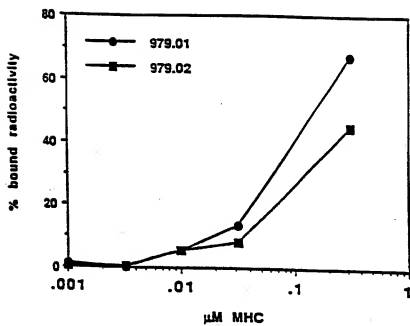
Figure 12

A24 Inhibition.

Figure 13

Figure 14A

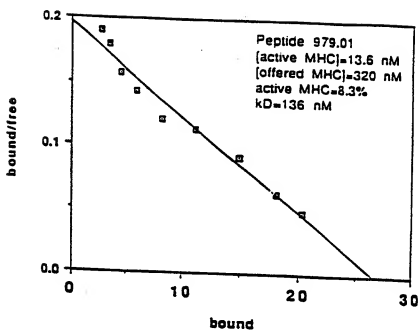Peptide 979.01
[active MHC]=13.6 nM
[offered MHC]=320 nM
active MHC=8.3%
kD=136 nM

Figure 14B

Peptide 979.02
[active MHC]=23.6 nM
[offered MHC]=320 nM
active MHC=7.4%
kD=28 nM
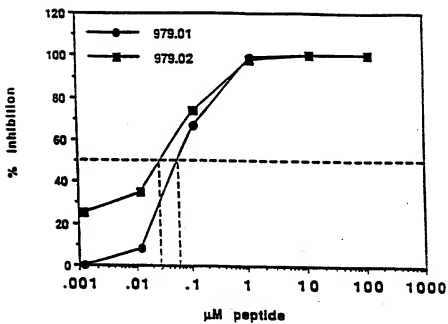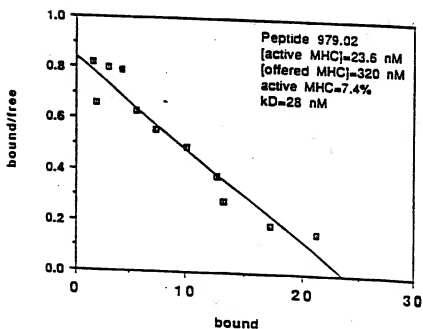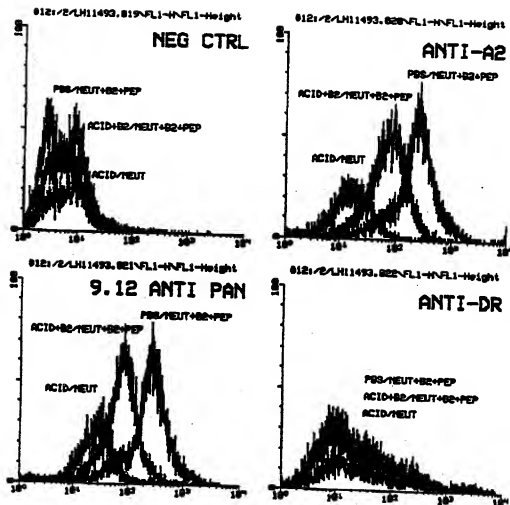
Figure 15

Figure 16

CTL INDUCTION
GC43 A2.1 CD4 DEPLETED RESPONDER
777.03 + 924.07 + 927.32 POOL

y-axis: % Release

x-axis: E: T ratio

- ● ─ Acid Strip PBMC + peptide
- ○ ─ Acid Strip PBMC - peptide
- ■ ─ Acid Strip PHA Blasts + peptide
- □ ─ Acid Strip PHA Blasts - peptide

Figure 17

Figure 18

CTL INDUCTION
GC49 A2.1 CD4 DEPLETED RESPONDERS
939.03 PEPTIDE

■ Acid Strip PHA Blasts + peptide   □ Acid Strip PHA Blasts - peptide
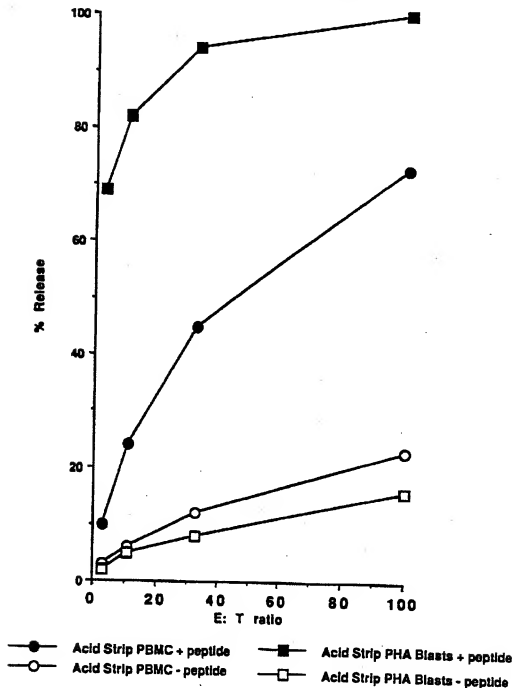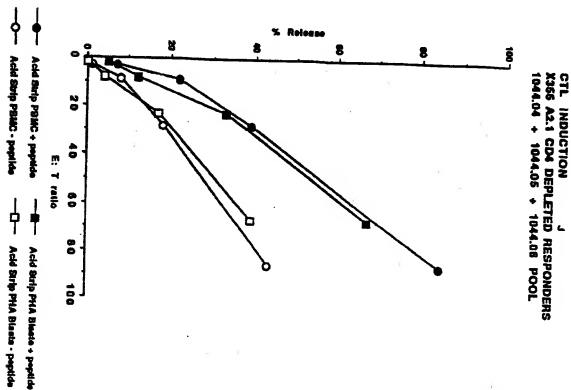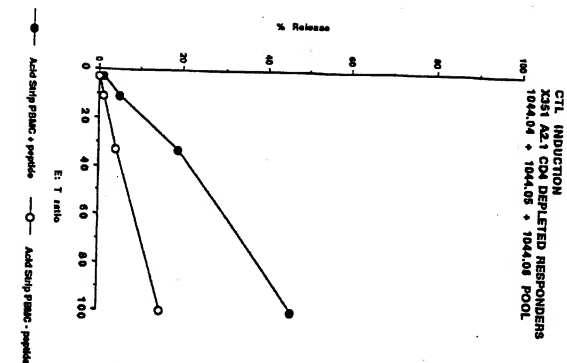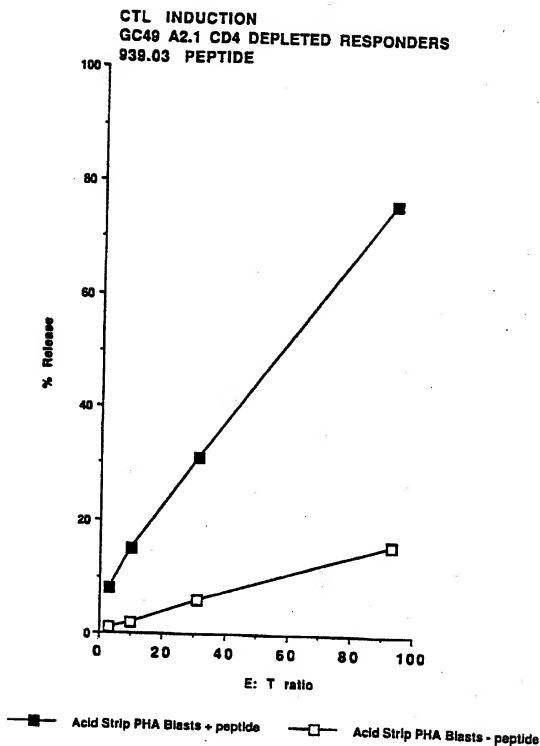
Figure 19

MAGE1 Peptide Specific, HLA-A1-Restricted CTL Can Kill Melanoma Cells Expressing the Endogenous Antigen

% Specific Cytotoxicity

CTL Line 1 (GC66.1)

CTL Line 2 (GC66.2)

Effector/Target
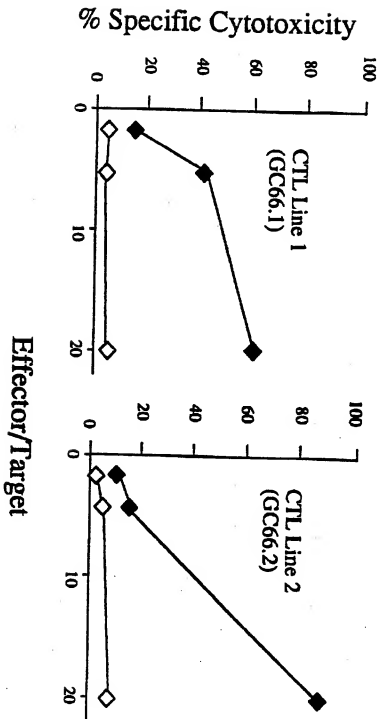
◆ 938-mel (HLA-A1/24, MAGE1+)

◇ 888-mel (HLA-A1/24, MAGE1−)

Figure 20

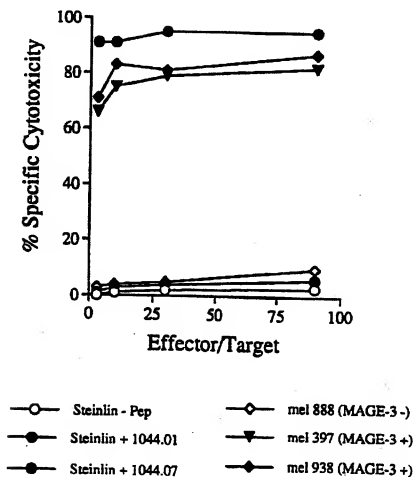## Cytotoxic Activity of an HLA-A1-Restricted CTL Line Specific for a MAGE3 Peptide (1044.07)

Legend	
—○— Steinlin - Pep	—◇— mel 888 (MAGE-3 -)
—●— Steinlin + 1044.01	—▼— mel 397 (MAGE-3 +)
—●— Steinlin + 1044.07	—◆— mel 938 (MAGE-3 +)

Induction Using Different Methods to Load Peptides onto SAC-I Cells.

Donor GC164
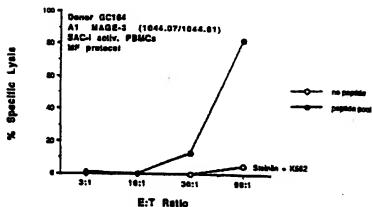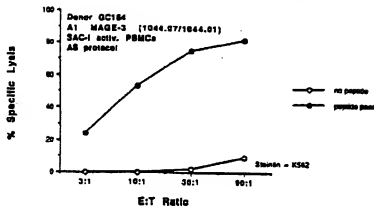A1 MAGE-3 (1044.07/1044.01)
SAC-I activ. PBMCs
AB protocol

○ no peptide
● peptide pool

% Specific Lysis

E:T Ratio   3:1   10:1   30:1   90:1

Staphn - K562

Donor GC164
A1 MAGE-3 (1044.07/1044.01)
SAC-I activ. PBMCs
MF protocol

○ no peptide
● peptide pool

% Specific Lysis

E:T Ratio   3:1   10:1   30:1   90:1

Staphn - K562

Donor GC164
A1 MAGE-3 (1044.07/1044.01)
SAC-I activ. PBMCs
Pulse only (no MF or AB protocol)

○ no peptide
● peptide pool

% Specific Lysis

E:T Ratio   3:1   10:1   30:1   90:1

Staphn - K562

Donor GC164
A1 MAGE-3 (1044.07/1044.01)
SAC-I activ. PBMCs
Dump (no MF or AB protocol)

○ no peptide
● peptide pool

% Specific Lysis

3:1   10:1   30:1   90:1

Staphn - K562

Figure 22
Peptide Screening:
MAGE/A11

Donor GC210
MAGE/A11
Pool 1

% Specific Lysis

E:T Ratio

BVR + K562

- no peptide
- 1072.13
- 1072.18
- 1072.20

Donor GC210
MAGE/A11
Pool 3

% Specific Lysis

E:T Ratio

BVR + K562

- no peptide
- 1072.39
- 1072.22
- 1072.15

Figure 23

Peptide Screening:
HIV/A3

Donor X297
HIV/A3
Pool 1

○ — no peptide
● — 1069.42
■ — 1069.43

EHM + K562

% Specific Lysis

E:T Ratio

Donor X297
HIV/A3
Pool 3

○ — no peptide
● — 1069.46
■ — 1069.47
▲ — 1069.48

EHM + K562

% Specific Lysis

E:T Ratio

Figure 24

Peptide Screening:
HCV/A3

Donor GC2
HCV/A3
Pool 1

% Specific Lysis

E:T Ratio

○ no peptide
● 1073.10
■ 1073.11
▲ 1073.16

EHM + K562

Figure 25

**Peptide Screening:
HBV/A3**

Donor GC220
HBV/A3
Pool 1

% Specific Lysis

EHM + K562

E:T Ratio

○ no peptide
● 1069.15

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/00; C07K 7/06, 7/08
US CL : 424/88; 514/2; 530/300, 327, 328
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88; 514/2; 530/300, 327, 328

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Exp. Med., vol. 174, number 3, issued 01 September 1991, Romero, et al., "H2K-d-restricted antigenic peptides share a simple binding motif", pages 603-612, see especially the abstract.	1-20
Y	Nature, vol. 353, issued 26 September 1991, T. S. Jardetzky, et al., "Identification of self peptides bound to purified HLA-B27", pages 326-329, see entire document.	1-20
Y	Nature, vol. 351, issued 23 May 1991, K. Falk, et al., "Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules", pages 290-296, see abstract.	1-20

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be part of particular relevance
"E"	earlier document published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 November 1993	1 7 NOV 1993

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	THOMAS CUNNINGHAM
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